Proceedings
# of the
Society
for
Experimental Biology and Medicine

INCLUDING THE FOLLOWING SECTIONS

CLEVELAND, O.
DISTRICT OF COLUMBIA
ILLINOIS
IOWA
MINNESOTA
MISSOURI
NEW YORK
PACIFIC COAST
PEIPING, CHINA
SOUTHERN
SOUTHERN CALIFORNIA
WESTERN NEW YORK
WISCONSIN

VOLUME 45

OCTOBER-DECEMBER, 1940 (INCLUSIVE)

NEW YORK

m

Press of
THOMAS J. GRIFFITHS SONS, INC.
Utica, N. Y.

CONTENTS

SCIENTIFIC PROCEEDINGS, VOLUME 45:

Page

Two hundred and eighty-third issue, October, 1940, No. 1 1

Two hundred and eighty-fourth issue, November, 1940, No. 2 517

Two hundred and eighty-fifth issue, December, 1940, No. 3 759

Authors' Index (Volume 45) 877

Subject Index (Volume 45) 883

Digitized by the Internet Archive
in 2024

https://archive.org/details/experimental-biology-and-medicine_1940-10_45_1

Proceedings

of the

Society

for

Experimental Biology and Medicine

Vol. 45. October, 1940. No. 1.

SECTION MEETINGS

DISTRICT OF COLUMBIA	
U. S. Public Health Service	June 11, 1940
MISSOURI	
Washington University Medical School	October 9, 1940

11563

Clinical Experiences with the Measurement of the Urinary Excretion of Vitamin B_1.

George A. Carden, William D. Province and Joseph W. Ferrebee.* (Introduced by W. W. Palmer.)

From the Departments of Medicine and Neurology, College of Physicians and Surgeons, Columbia University, the Presbyterian Hospital and the Neurological Institute, New York City.

Vitamin B_1 is currently used in the treatment of a large number of complaints and diseases. In relatively few of these situations has an actual deficiency of vitamin B_1 been demonstrated in the patients. In the majority of instances the presence of a deficiency is assumed because of resemblance between the symptoms under suspicion and the symptoms which occur in known vitamin B_1 deficiencies, such as beri-beri and certain forms of polyneuritis. At times the diagnosis may be corroborated by the patient's previous dietary history and by an improvement in his symptoms coincident with vitamin B_1 therapy. However, evaluation of dietary histories and therapeutic responses is difficult, and if direct measurements of some phase of vitamin B_1 metabolism can be correlated with the patient's need for vitamin B_1, the problem of vitamin B_1 therapy might be considerably simplified. The data presented in this paper furnish further evidence[1, 2, 3] that such a correlation may be possible.

---

* The authors wish to express their appreciation of the technical assistance given them by Miss Vera James.

Method. We have measured the urinary excretion of vitamin B_1 using a modification of the procedure of Hennessey and Cerecedo[4] arranged to be convenient for clinical use.[5] The method gave results which were comparable to those obtained by Melnick and Field[6] on the same samples of urine.†

Results. The amount of vitamin B_1 appearing in 24-hour urine samples is generally agreed to be some function of the amount of vitamin B_1 ingested in that 24-hour period.[1, 2, 7] Normal individuals on ordinary normal diets appear to excrete somewhere between 10 and 40% of the amount of B_1 in their diet. The addition of small amounts of vitamin B_1 to these normal diets or the injection of vitamin into these normal individuals results in the excretion of about 10-40% of this added B_1, Table I. This increased B_1 excretion

TABLE I.
Excretion of Vitamin B_1 by Normal Individuals (Interns) on Their Usual Diets and Their Response to a Tolerance Test Dose.

Subject	24-hour excretion of B_1 on usual normal diet	24-hour excretion of B_1 on usual normal diet + 0.5 mg of B_1 subcutaneously
	γ	γ
P	108	163
C	150	252,238
D	120	
I	220	
A	77*	198
S	283	
N	149	230
R	168	335
W	109	280
Wl	260	—
D	390	avg increment 133
Pr	153 (after 1 mg po. 258)	
	Avg 182	

*Ulcer subject.

---

[1] Westenbrink, H. G. K., and Goudsmit, J., *Arch. Neérl. de physiol.*, 1938, **23**, 79.

[2] Robinson, W. D., Melnick, D., and Field, H., Jr., *J. Clin. Invest.*, 1940, **19**, 399.

[3] Hou, H. C., and Yang, E. F., *Chinese J. Physiol.*, 1939, **14**, 269.

[4] Hennessey, D. J., and Cerecedo, L. R., *J. Am. Chem. Soc.*, 1939, **61**, 179.

[5] Ferrebee, J. W., and Carden, G. A., *J. Lab. and Clin. Med.*, 1940, **25**, 1320.

[6] Melnick, D., and Field, H., Jr., *J. Biol. Chem.*, 1939, **127**, 515.

† The writers wish to thank Dr. H. Field, Jr., for his kindness in sending them these urine samples.

[7] Westenbrink, H. G. K., and Goudsmit, J., *Arch. Neérl. de physiol.*, 1937, **22**, 319.

can be considered their response to a tolerance test dose of vitamin B_1. Since no effort was made in our series to control the dietary selection or intake in these normal subjects, considerable variation may have occurred in the amount of B_1 ingested by the same individual on different days, and as a result it is not possible to determine from our data the exact percentage of the added vitamin which these individuals excreted. The essential point at present is that the normal individuals on normal diets had an excretion of vitamin B_1 of more than 100 γ per 24 hours and that this excretion could be routinely augmented by the administration of a small tolerance dose.[1, 2, 7] On the other hand, the 24-hour urinary excretion of vitamin B_1 in a group of hospital patients suspected of suffering from vitamin B_1 deficiency was low, Table II. These patients had been on apparently deficient diets for varying periods of time preceding hospital admission and in some there were clinical symptoms suggestive of vitamin B_1 deficiency. When small test doses of vitamin B_1‡ were administered to the members of this group, approximately normal increments in the urinary excretion of vitamin B_1 were obtained in only a portion of the cases, 1-4, Table III. In the others, 5-9, the patients in whom the evidence for B_1 deficiency was strongest, the responses became normal only after the patient had been in the hospital for several days, received B_1 in several tolerance tests, and eaten an adequate diet.

Discussion. We have determined the urinary excretion of vitamin B_1 directly by a chemical method and have tried to correlate its urinary excretion with the state of vitamin B_1 nutrition. In so doing we appreciate the numerous variables of tissue saturation and renal function which must be considered in interpreting measurements of

TABLE II.
The 24-hour Urinary Excretion of Vitamin B_1 by Hospital Patients Suspected of Vitamin B_1 Deficiency.

Subject	24-hour excretion of vitamin B_1 γ
Br	40
McK	15
Sh	70
Wd	25
Di	50
Ad	20
Dy	15
Lm	15
Lv	5
Dt	15
Pl	20

---

‡ Supplied by Roche-Organon Co., Nutley, N. J.

TABLE III.

Effect of Small Doses of Vitamin B_1 upon the Urinary Excretion of Vitamin B_1 in Patients Suspected of Vitamin B_1 Deficiency.

Subject		Diagnosis	Daily vitamin B_1 treatment	Amt of vitamin B_1 in 24-hour urine sample γ
1	Br		0	40
			.5 mg p.o.	130
			”	110
			.5 mg i.m.	100
			”	150
			”	150
			”	80
			”	80
			”	130
			0	15
			1 mg p.o.	200
2	McK	Chronic cholecystitis, healed or inactive ulcerative colitis	0	15
			.5 mg i.m.	110
			”	200
			”	140
			”	215
			0	110
			1 mg p.o.	210
3	Di	Addison's disease	0	50
			1 mg s.c.	380
			”	310
			1 mg p.o.	190
			”	190
4	Ad		0	15
			1 mg s.c.	120
			”	130
			”	230
5	Dt	Ulcerative colitis	0	15
			.5 mg i.m.	50
			”	50
			1 mg i.m.	140
			”	190
6	Am	Cardiac insufficiency of unknown etiology	1 mg s.c.	15
			”	40
			”	190
			”	160
			”	120
7	Pl	Pernicious anemia	.5 mg s.c.	20
			”	10
			”	15
			1 mg s.c.	50
			”	140
			”	140
			”	140
8	Lm	Advanced carcinoma with metastases	0	15
			1 mg i.m.	70
			”	90
			”	80
			”	45
			”	130
			20 mg i.m.	2000+
9	Dy	Alcoholic polyneuritis	0	15
			”	15
			.5 mg p.o.	20

Subject	Diagnosis	Daily vitamin B_1 treatment	Amt of vitamin B_1 in 24-hour urine sample γ
		.5 mg i.m.	50
		”	25
		”	50
		”	30
		”	50
		1 mg i.m.	50
		”	120
		0	15
		1 mg i.m.	60
		2 mg p.o.	160
		”	340
		0	75
		”	70
		”	15

urinary excretion of vitamin. At present it seems evident that patients who have been on low vitamin B_1 diets excrete less than the usual normal amounts of vitamin B_1 in their urine, and that the more deficient ones have a less than normal increment in B_1 excretion following the administration of small doses of vitamin. A much larger series of patients and a better understanding of the factors relating tissue saturation and urinary excretion will be required before accurate interpretation of the results of B_1 tolerance tests can be attempted. However, it is interesting that in one year we were unable to find any patient whose urinary excretion of vitamin B_1 could not be raised to normal by the administration of very small amounts of vitamin, amounts comparable to those obtainable in ordinary normal diets.

Conclusion. It appears possible to correlate the urinary excretion of vitamin B_1 with the state of vitamin B_1 nutrition in man using a thiochrome method for determining the amount of vitamin B_1 in urine.

11564

Gravimetric Intestinal Oncometry in the Dog.

HAMPDEN LAWSON.

From the Department of Physiology, University of Louisville School of Medicine, Louisville, Ky.

The usual difficulties of quantitative plethysmography are multiplied when the method is applied to the intestine by the occurrence of intestinal contractions which may compress gases trapped within the lumen of the gut, even though the ends of the loop are open, and which, if strong enough, may pull mesentery into the chamber. These difficulties are obviated by the substitution of a simple gravimetric method, which permits, as an added advantage, simultaneous recording of intestinal motility.

A loop 6 to 10 cm in length, whose mesentery is long, is separated by incisions which are converged to the root of the mesentery, care being taken to preserve the loop's blood supply. With the dog lying supine, the loop and its prepared pedicle are lifted over a horizontal rod held in a clamp as close as possible to the root of the pedicle, and the pedicle is sutured to the rod (Fig. 1, a). The loop is threaded upon a light supporting rod (b), and the ends securely fastened. The projecting ends of the supporting rod are attached by means of threads to the horizontal beam of a spring balance (c). The tension of the spring and the leverage are adjusted until the loop on its rod, and the entire pedicle are swung clear of the animal. The loop should lie approximately in the same horizontal plane as the rod which supports the pedicle at the root.

It has been found simpler to record the movements of the horizontal beam indirectly, through a second light magnifying lever (e) as this permits adjustment of leverages without disturbing simultaneous

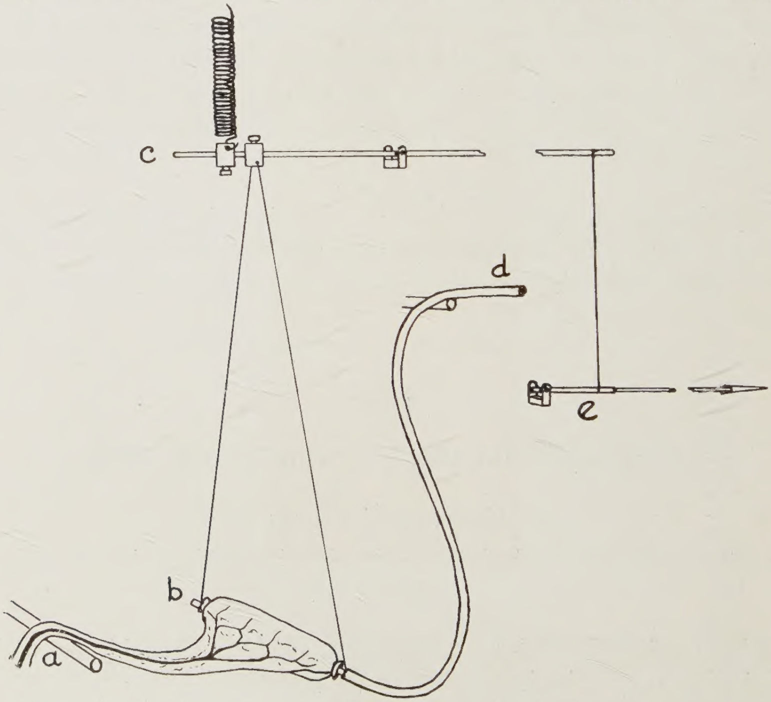


FIG. 1.
For description see text.

ordinates on the record. If long loops are taken, strong contractions may exert pull on the pedicle, and so interfere with the record. This does not occur if the loops are short, the ends are tied securely to the supporting rod, and the pedicle is slack. The vertical component in horizontal forces acting on the pedicle is reduced by having the threads connecting the loop with the beam of the balance long.

By using jeweled bearings in both fulcra, and long flexible helical springs, it is possible to obtain an apparatus which is sensitive to sudden changes of 0.1 g in the weight of the gut, and which returns to the base line when unloaded. With greater sensitivity than this, the base line tends to shift. A satisfactory spring may be made from 26 gauge steel wire, wound to have a diameter of 1 cm, a length of 10 cm. Each preparation is calibrated during the experiment by laying weights on the intestine while it is in recording position.

With low magnification in the 2 levers and fairly high natural frequency for the apparatus, the pulse may be recorded ballistically. Usually, however, the pulse does not show in the record.

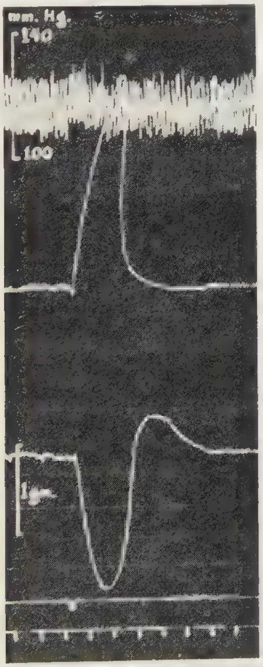


Fig. 2.

Records from above down are: carotid arterial pressure; balloon record of loop, taken from large-bore water manometer with volume recorder; weight of loop. At the signal 10 gamma epinephrine were injected into the superior mesenteric artery, producing strong motor response of the loop. Time in minutes. The dilator rebound in weight following epinephrine is common but not constant.

Simultaneous records of intestinal motility may be made by substituting for the rod which supports the loop a light metal tube carrying a condom balloon. One projecting end of the tube is closed, the other connected through very flexible rubber tubing (d) to a water manometer or other recording device. Balloon and tubing are filled with air. The tubing is supported in a long loop as it leaves the intestine, so as to damp the movements of the weight recorder as little as possible.

The loop hanging in air is coated with liquid petrolatum to limit drying. It is kept close to body temperature with a three-sided metal shield with open top which encloses the lower half of the animal and in which lamp bulbs are hung as heating elements.

Under some conditions accumulation of secretions in the loop causes a progressive increase in weight throughout the period of observation. It is simpler to work on a steadily rising weight record than to prevent this with drainage. The spring tension may be adjusted from time to time to keep the writing lever in recording position.

Figure 2 shows the type of record obtained with this arrangement. Under optimum conditions, with the gut showing strong rhythmic contractions, rhythmically recurrent changes in weight are recorded, reciprocating with the motility record. These are just visible in the weight record in Fig. 2, during the periods when the motility record shows weak rhythmic contractions.

11565

Effect of Morphine Sulfate on Serum Choline-Esterase.

DONALD SLAUGHTER AND ROBERT W. LACKEY.

From the Laboratory of Physiology and Pharmacology, Baylor Medical School, Dallas, Texas.

It has been previously reported by Slaughter and Gross,[1] and Slaughter and Munsell[2] that physostigmine and prostigmin respectively potentiate the action of morphine on the intestine, blood pressure, on toxicity and pain. The belief that these effects might be in part due to a depression of choline-esterase by morphine was expressed.

---

[1] Slaughter, Donald, and Gross, E. G., *J. Pharm. and Exp. Therap.*, 1940, **68**, 96.

[2] Slaughter, Donald, and Munsell, Donald W., *J. Pharm. and Exp. Therap.*, 1940, **68**, 104.

Bernheim and Bernheim[3] have shown that morphine depresses the brain choline-esterase *in vitro* They used a biological method, employing the guinea pig intestine to demonstrate these effects. Having at hand a series of dogs which had had weekly determinations of serum choline-esterase done for a period of one year, we made a series of *in vitro* tests using morphine sulfate by incubating ½ cc of serum for 15 minutes with 1 mg of morphine sulfate, after which the esterase activity was determined by the method of Hall and Lucas.[4]

After repeated trials, we were unable to confirm the results of Bernheim and Bernheim as regards the *in vitro* depressant effect of morphine sulfate on choline-esterase.

Despite the negative results from experiments *in vitro,* we believed that morphine did have an effect on the acetylcholine-choline-esterase system *in vivo.* Experiments were carried out on a series of 6 dogs: a 4 cc sample of blood was drawn by venipuncture, 5 mg per kilo of morphine sulfate was injected subcutaneously, and after 20 minutes another sample of blood was obtained. The 20-minute interval was

TABLE I.
Serum Choline-esterase in Dogs Before and 20 Minutes After Injection of 5mg/kg of Morphine Sulfate.

Normal	20 min after injection	Difference	Normal	20 min after injection	Difference
Dog 4			Dog 9		
1.84	0.84	1.00	1.95	1.55	.40
1.77	1.48	.29	1.84	1.64	.20
1.67	1.20	.47	1.57	1.45	.12
1.47	1.17	.30	1.68	1.43	.25
1.44	1.05	.39	1.84	1.30	.54
1.70	1.37	.33	1.78	1.60	.18
Dog 7			Dog 10		
1.47	1.26	.21	1.71	1.34	.37
1.85	1.43	.42	1.80	1.55	.25
1.83	1.45	.38	1.50	1.25	.25
1.63	1.31	.32	1.84	1.50	.34
1.54	1.10	.44	1.93	1.31	.62
1.82	1.38	.44	1.96	1.67	.29
Dog 8			Dog 11		
1.85	1.65	.20	2.05	1.97	.08
2.14	1.99	.15	2.48	2.00	.48
2.25	1.93	.32	2.26	2.02	.24
1.93	1.37	.56	2.65	2.04	.61
2.38	1.73	.65	2.76	1.70	1.06
2.35	1.73	.62	2.90	2.60	.30
		Mean values	1.927	1.536	0.391

---

[3] Bernheim, F., and Bernheim, M. L. C., *J. Pharm. and Exp. Therap.*, 1936, **57**, 427.

[4] Hall, G. E., and Lucas, C. C., *J. Pharm. and Exp. Therap.*, 1937, **59**, 34.

chosen since preliminary work indicated that depression of choline-esterase activity is most marked at that period. Results are shown in Table I.

To obviate errors in the above series, determinations were made concurrently in which morphine sulfate was added to the blood *in vitro*. In other instances blood samples were drawn at 20-minute intervals to see whether repeated punctures with the needle would cause variations in esterase activity. In 10 experiments *in vitro* the mean change in cholin-esterase activity was (–) 0.10 units; in 6 determinations repeated at 20-minute intervals (using blood withdrawn without the giving of any drug) the mean change in esterase activity was zero. While the differences between the normal values for serum choline-esterase and those following the injection of morphine sulfate are not great (20%), we feel that they are significant because of their consistency.

Summary and Conclusions. 1. Morphine sulfate added to blood *in vitro* produces no consistent change in choline-esterase activity. 2. Serum choline-esterase activity in the dog is consistently lowered following the injection of morphine sulfate. 3. The *in vivo* effect of morphine on serum choline-esterase furnishes another indication that morphine acts through a cholinergic mechanism.

11566

Response of Sex Characters of the Adult Female Starling to Synthetic Hormones.*†

EMIL WITSCHI AND N. W. FUGO.

From the Department of Zoology, State University of Iowa, Iowa City, Iowa.

The female sex of birds is often remarkable for extensive ambisexual features. In the starling *(Sturnus v. vulgaris)* the females have paired vasa deferentia which enlarge during the breeding season and, as in males, the bills are yellow during the first 6 months of the year. In a previous paper[1] it was shown that bill color as well

---

* Aided by a grant from the Committee for Research in Problems of Sex, The National Research Council.

† The authors are obliged to the following, who have supplied the hormone preparations: Parke, Davis and Co.: estrone (Theelin), estriol (Theelol) and estradiol (Dihydrotheelin); Ciba Pharmaceutical Products, Inc.: Stilbestrol, progesterone (Lutocylin), androsterone, testosterone propionate (Perandren).

[1] Witschi, Emil, and Miller, R. A., *J. Exp. Zool.*, 1938, **79**, 475.

as size of the wolffian ducts respond to androgens and that during the breeding season the females of this species release considerable amounts of androgenic hormone. The present series of experiments was planned to decide whether in this interesting case of hormonal ambisexuality and morphological pseudohermaphrodism male and female sex hormones are pitted against each other, forming a system of dynamic balance, or whether the female organism has become adapted in some other way to the presence of hormones of contrary sex type. In the interest of clearness of results this work was started with synthetic sex hormones. It was therefore only natural to broaden its scope by testing also some substances which do not occur or function in the normal organism as androgens or estrogens.

The results of the injection series are summarized in the 2 tables. About half of the females were castrated, though in 3 the ovarial tissues were found incompletely removed. Except for one series (Table II) the experiments were carried out during the quiescent season and always with birds that had been in captivity for a sufficiently long time to insure a relative state of inactivity of the sex organs. The ovary of untreated controls weighs under these conditions from 10 to 35 mg, the left oviduct from 25 to 50 mg and the right vas deferens (from the lower edge of the kidney to its entrance into the cloaca) from 1 to 2.5 mg. In castrates the oviducts shrink even more, weighing often less than half the minimal weight of quiescent normals (all weights given in this paper are of Bouin fixed and alcohol preserved material). In most cases the daily dose was given 10 times on consecutive days. It seems that after the first week no further increase is obtained with a constant hormone level.[2]

From our Table I it can be seen that estradiol, estrone, estriol and stilbestrol stimulate exclusively the oviducts (the rudiment on the right side also becomes enlarged). The doses given are in some instances excessive and produce oviducts nearly as large as those of wild birds killed at the height of the breeding season. In the latter the oviducts average about 3000 mg, the heaviest observed weighing 3306 mg. Since extraction of the whole bodies of birds in early breeding condition yields only small amounts of sex hormones, it appears that the injected preparations are not being economically used.

Progesterone has no influence on either the female or the male sex characters. More extensive series with sparrows, which will be described elsewhere, prove that even in combination with estrone the progesterone fails to contribute to the response.

---

[2] Keck, W. N., *J. Exp. Zool.*, 1934, **67**, 315.

TABLE I.

Hormone reactions in female starlings injected during the quiescent season. c, castrate; i, incomplete castrate. In the weight of the right vas deferens only the part from the lower edge of the kidney to the cloaca is included. In the last group estrone was injected 10 times, testosterone only 7 times, during the first 7 days. Weights from material preserved in alcohol.

Bird	Hormone		Ovary		Oviduct		Right vas def.	
	Name	Daily dose γ	wt, mg	type	wt, mg	type	wt, mg	type
68 c	estradiol	0.6	—		35	0-I	(ca. 1.3)	0
79 c	estriol	100	—		110	II	(ca. 1.3)	0
57 c	estrone	5	—		149	II	(ca. 1.3)	0
232	”	50	17	0	110	II	1.4	0
233	”	50	18	0	182	II	1.7	0
228 i	”	50	—		198	II	1.6	0
231	”	500	22	0	579	II	1.0	0
178 i	”	500	—		1681	III	1.6	0
312	stilbestrol	50	15	0	1112	III	1.9	0
310	”	50	24	0	1471	III	1.3	0
306	”	50	32	0	1533	III	1.4	0
311	progesterone	500	30	0	50	0	1.9	0
227 i	”	500	—		34	0	1.6	0
56 c	androsterone	330	—		10	0	(ca. 8)	I
226 c	”	3000	—		114	II	14.1	III
80 c	testosterone propionate	100	—		16	0	(ca. 8)	I
182 c	” ”	1250	—		241	II	11.5	III
302	” ”	1250	19	0	91	I	14.3	III
303	” ”	1250	30	0	82	I	11.3	III
301	” ”	1250	15	0	559	II	14.4	III
234	estrone + testosterone prop.	50 + 1000	37	0	652	II	(ca. 10)	II-III
140 c	” ” ”	50 + 1000	—		633	II	9.3	II
83 c	” ” ”	50 + 1000	—		1536	III	10.1	III
Controls (range)	—		15-35	0	25-50	0	1-2	0
Castrates ”	—		—		5-20	0	1-1.5	0

TABLE II.
Reactions Produced by Testosterone Injections into Captive Female Starlings in the Month of May.

Bird	T. prop. Daily dose, γ	Ovary		Oviduct		Right vas def.	
		wt, mg	Type	wt, mg	Type	wt, mg	Type
Control 1	0	70	I	99	I	1.7	0
” 2	0	93	I	182	II	1.9	0
313	10,000	36	0	1144	III	12.4	III
316	10,000	40	0	851	III	13.9	III
307 c	10,000	—		716	III	14.8	III
305 c	10,000	—		1024	III	12.8	III

In contrast to the selective character of the gynogenic substances the androgens (androsterone and testosterone propionate) stimulate all secondary sex characters. However, the effects appear at different quantitative levels. Unpublished experiments with castrate male starlings show that the bill color has a lower threshold value than the deferent ducts (about 1 γ and 20 γ of testosterone propionate per day). The oviduct becomes stimulated only by doses well above 330 γ of androsterone or 100 γ of testosterone. Daily doses of 1.25 and 10 mg of testosterone produce oviducts of a size characteristic for birds approaching the breeding condition (early April) and vasa deferentia about twice the weight they ever attain in breeding females. The ovaries of the starling remain quiescent though in sparrows the ovaries also become stimulated (unpublished). In all groups the histological structure of the enlarged oviducts is identical with that of breeding females, no matter whether androgens or gynogens were used to stimulate their development.

It appears that the presence or absence of the ovary has no influence on the extent of development of the ducts in the injected birds, at least not at the levels of hormone dosages used in the present set of experiments. The data of Table II indicate, however, a depressive action on ovarial development. This latter series was made during the spring breeding season with birds that had been caged in the laboratory since early spring (February 10). Birds in captivity are crowded and without accommodations for nest building. Their ovaries enlarge only to a modest degree and spontaneous ovulation never occurs. In birds in the open, the ovaries and oviducts have increased in weight up to 100 and 150 times by late April while in those in captivity the increment is always less than ten fold, even one month later. The results presented in Table II suggest that the injected testosterone has inhibited the gonadotropic activity of the hypophysis. The slightly greater average weight of the oviducts in the non-castrated females is either due to the advantage which they had from the beginning of the experiment or purely accidental. In a forthcoming paper it will be shown that the sparrow differs distinctly in the reactions to androgens, in that its ovaries are stimulated to grow and produce hormones.

Discussion. The literature of recent years contains numerous reports on gynogenic properties manifested by "androgens". In most instances the reactions were obtained by administration of large amounts of the male hormone in animal species where the normal female does not usually exhibit any evidences of the presence of considerable amounts of this hormone. In the starling, however, it seems quite possible that androgens normally contribute to the development of the oviduct. Our series of combined estrone and testosterone injections prove not only that there is no antagonism between these hormones but that they coöperate in the stimulation of the oviduct (Table I). In view of the established presence of male hormones in breeding female starlings there remains little doubt but that the 2 sex hormones also coöperate under normal conditions in the spring development of the oviducts. Since female birds exhibit many ambisexual features, the responsiveness of the oviduct to androgens may have developed as a special adaptation to this condition. The remarkable and strictly specific gynogenic potency of stilbestrol remains without explanation. While this synthetic substance seems to be capable of inducing all the gynogenic reactions of natural female sex hormones in all classes of vertebrates, its faculty of imitating testosterone and the other ambigenic "male" hormones in the starling remains limited to the female component.

Summary. Adult female starlings in the quiescent season are a favorable material for the study of the biological properties of natural and synthetic sex hormones. Their ovaries and oviducts are capable of increasing in weight 100 to 150 times and the wolffian ducts 10 to 20 times. The bill color and the wolffian ducts react only on stimulation by androgens while the oviducts react in an identical way on either male or female hormones. Stilbestrol produces purely gynogenic effects and is considerably more potent than equal doses of estrone. Progesterone even in high doses or in combination with estrone gives no effects; it does not seem to be one of the hormones that take a part, normally, in the endocrine physiology of passerine birds.

11567

Acute Toxicity of Sodium Salts of Sulfapyridine, Sulfathiazole and Sulfamethylthiazole.*

David Lehr, William Antopol, Jacob Churg and Helmuth Sprinz.

From the Laboratories of the Newark Beth Israel Hospital, Newark, N. J.

Comparing the acute toxicity of the sodium salts of sulfapyridine, sulfathiazole and sulfamethylthiazole in mice, Barlow and Homburger[1] found that sulfapyridine administered orally appeared to be considerably more toxic than the thiazole derivatives (3.4 times as toxic as sulfamethylthiazole, and 4.57 times as toxic as sulfathiazole). Long, Haviland and Edwards,[2] using the subcutaneous route for determination of the acute toxicity of the same compounds, reported sodium sulfamethylthiazole (LD_{50}0.86 g/kg) to be more toxic than sodium sulfapyridine (LD_{50}1.0 g/kg), while sodium sulfathiazole was found to be about half as toxic as the latter drug (LD_{50}1.95 g/kg). Van Dyke, Greep, Rake and McKee,[3] in similar experiments placed the LD_{50} for sodium sulfapyridine at about the same level (0.95 g/kg), whereas the LD_{50} of sodium sulfathiazole was found to be at 1.45 g/kg. They made the interesting observation that with similar doses (0.5 g/kg) both compounds were equally toxic, while after administration of large amounts (1.0 g/kg), sodium sulfapyridine appeared to be considerably more toxic. Fifty percent of the mice receiving sulfapyridine died within 3 hours, whereas none of the mice receiving sulfathiazole died spontaneously at the end of 6 hours. Since the blood concentrations of both compounds were found to be about the same within the aforementioned time interval, the early death after sodium sulfapyridine could not be correlated with the level of the drugs in the blood. Long and coworkers[2] stressed the fact that the oral route of administration is not valid for the assay of the acute toxicity of poorly soluble derivatives of sulfanilamide. They pointed out that the solutions of the sodium salts are very alkaline (pH 10 to 11) and the sodium ion therefore is split off in blood and tissues; hence by means of the parenteral

---

* Aided by a grant from the Sidney C. Keller Research Fund.

[1] Barlow, O. B., and Homburger, E., Proc. Soc. Exp. Biol. and Med., 1940, **43**, 317.

[2] Long, P. H., Haviland, J. W., and Edwards, L. B., Proc. Soc. Exp. Biol. and Med., 1940, **43**, 328.

[3] van Dyke, H. B., Greep, R. O., Rake, G., and McKee, C. M., Proc. Soc. Exp. Biol. and Med., 1939, **42**, 410.

route the true toxicity of the poorly soluble parent drug can be determined with accuracy.

Potentiometric titration of the sodium salts of the 3 compounds, however, showed that precipitation occurs after a slight decrease of pH and is complete at pH 9 for sodium sulfapyridine and at pH 7 for sodium sulfathiazole and sodium sulfamethylthiazole (Lott and Bergheim,[4] Fosbinder[5]). It must be assumed, therefore, that after subcutaneous injection of the sodium salt the parent drug is precipitated out almost completely in the subcutis and then absorbed at a rate depending on the solubility of the free compound. Since sulfathiazole is considerably more soluble, and sulfamethylthiazole somewhat less soluble than sulfapyridine, the different rates of absorption have to be taken into account after subcutaneous administration. (At 28°C 600 mg sulfathiazole, 280 mg sulfapyridine and 200 mg sulfamethylthiazole are soluble in one liter distilled water.)

To eliminate variations due to the different rates of absorption an attempt was made to determine the intravenous toxicity in rats and mice. However, despite very slow injection into the tail vein (10 to 15 minutes), the animals developed severe toxic symptoms; with higher doses death during injection could not be avoided even though the rate of injection was further reduced. The highest single dose tolerated, using a 2.5% solution and 15 minutes as the injection period, was 0.85 g/kg sodium sulfapyridine, 1.0 g/kg sodium sulfathiazole and 0.5 g/kg sodium sulfamethylthiazole.. Some data on the acute intravenous toxicity of sodium sulfapyridine were reported by Marshall and Long[6] for dogs and by Kohn-Richards[7] for rabbits. Kohn-Richards[7] found that 0.5 g/kg killed 25%, 0.8 g/kg approximately 70% of the rabbits, whereas 1.0 g/kg was always fatal.

As a consequence of our experience with intravenous administration, the intraperitoneal route was chosen for a study of the comparative toxicity of the 3 derivatives. Since absorption occurs easily from the large surface of the peritoneal cavity and the extent of absorption can be checked readily at autopsy, this mode of administration was believed to be more advantageous than the subcutaneous route.

Method. 208 male albino rats of uniform age and from a standard strain weighing between 150 and 200 g were used. The animals were kept on a standard diet (Purina Dog Chow Checkers) and were observed for 3 days following the injection. A 2.5 or a 5% solution of sodium sulfapyridine[†] was used and equimolecular amounts of sodium sulfathiazole and sodium sulfamethylthiazole were administered in the same volume of distilled water. Thus with the different derivatives, the animals received equal quantities of the sulfanilamide portion of the molecules (1.0 g sodium sulfapyridine monohydrate ≘ 1.025 g sodium sulfamethylthiazole ≘ 0.98 g sodium sulfathiazole). Animals sacrificed soon after intraperitoneal injection showed a thin milky layer of fine precipitate covering the entire surface of the abdominal cavity and its contents. Rats that did not die spontaneously within the 3-day period were sacrificed. Complete autopsies were performed on all animals and special attention was given to the presence of drug precipitation in the abdominal cavity, and to concrement formation in the urinary tract. All organs were fixed in 20% formalin for histological examination.

---

[4] Lott, W. A., and Bergheim, F. H., *J. Am. Chem. Soc.*, 1939, **61**, 3593.

[5] Fosbinder, R. J., personal communication.

[6] Marshall, E. K., Jr., and Long, P. H., *J. A. M. A.*, 1939, **112**, 1671.

[7] Kohn-Richards, R., *Proc. Am. Physiol. Soc.*, 52 Ann. Meeting, 103, March, 1940.

Results. The data dealing with the acute toxicity of the 3 compounds are summarized in Table I.

TABLE I.
Acute Toxicity of Sodium Salts of Sulfapyridine, Sulfathiazole, and Sulfamethylthiazole in Rats.
(Intraperitoneal injection of equimolar amounts.)

Compound	Dose, g/kg	No. of rats	No. dead	% dead (for 10 or more)	Died within hrs (mean value)	No. of dead with precipitation or concrement formation in urinary tract
Sodium	0.5	9	0			
Sulfapyridine	0.75	25	0	0		
	1.00	10	0	0		
	1.12	5	5		8	0
	1.25	15	12	80	9	0
	1.50	10	10	100	3	0
Sodium	0.5	5	0			
Sulfathiazole	0.75	13	1	8		1
(equimolar	1.00	10	1	10	40	1
to sodium	1.12	5	4		26	4
sulfapyridine)	1.25	20	20	100	11	20
	2.00	10	10	100	4	10
Sodium	0.5	16	3	19	58	2
Sulfamethylthiazole	0.6	10	1	10	24	1
(equimolar	0.65	5	5		26	5
to sodium	0.75	20	20	100	20	16
sulfapyridine)	1.00	10	10	100	15	7
	1.5	10	10	100	15	10

[†] We wish to thank the Maltbie Chemical Company for providing sulfathiazole, sulfamethylthiazole and the sodium salts of these compounds, and Merck & Co. for supplying us with sulfapyridine and sodium sulfapyridine.

It is noteworthy that sodium sulfapyridine appears to have the lowest toxicity, sodium sulfathiazole is slightly more toxic, while sodium sulfamethylthiazole is twice as toxic as sodium sulfapyridine. It may be seen from the table, however, that after higher doses of sodium sulfapyridine (1.12 and 1.25 g/kg) death occurs much earlier than after sodium sulfathiazole. Even sodium sulfamethylthiazole, which is considerably more toxic, requires a much longer time to produce death with the same concentration (*e.g.* 1.50 g/kg). There was a striking difference in the toxic symptoms following injection of sodium sulfapyridine and those resulting from the thiazole derivatives. Sodium sulfapyridine caused an increase in reflex excitability and muscular tone with spastic rigidity of all body muscles which became noticeable within a few minutes after the intraperitoneal injection. Twitching soon followed, which increasing in strength and severity, finally led to attacks of epileptiform convulsions. (A similar picture of intoxication was described for mice and dogs by Marshall, Bratton, and Litchfield,[8] and for mice by Barlow and Homburger.[1]) If death from asphyxia did not occur in this stage the animals recovered gradually.

The thiazole compounds on the other hand produced a state of extreme muscular weakness up to complete flaccidity of all body muscles. While this picture developed with sulfathiazole about 10 to 15 minutes after the administration, it occurred only several hours after the injection of sulfamethylthiazole. All 3 compounds caused severe respiratory disturbances, which may be ascribed, at least partly, to the injection of large amounts of alkali.

It seems important to stress the fact that the alkalinity of the sodium salts of sulfapyridine and the thiazole derivatives is different despite the presence of the same amount of sodium. A 2% solution of sodium sulfapyridine has a pH of 10.7, while both sodium sulfathiazole and sulfamethylthiazole in the same concentration have a pH of 9.4. Thus, "sulfathiazole is more strongly acidic" (Lott and Bergheim[4]). In addition, potentiometric titration reveals that the 3 derivatives possess a very high buffer capacity in the range of pH 9-10 for sodium sulfapyridine and of pH 7-9 for the sodium salts of the thiazole compounds. Thus, considerable amounts of acid are necessary for neutralization. It can, therefore, be expected that the injection of large amounts of the sodium salts will cause disturbances in the acid-base balance of the body, particularly the administration of sodium sulfapyridine with its high alkalinity.

---

[8] Marshall, E. K., Jr., Bratton, A. C., and Litchfield, J. T., *Science*, 1938, **88**, 597.

Marked peritoneal irritation, with the presence of blood-tinged fluid in the abdominal cavity, was observed after injection of sulfapyridine and sulfamethylthiazole. Sulfathiazole caused less irritation though usually large amounts of clear exudate were found both in the pleural and peritoneal cavities.

Animals that survived after 24 hours showed no precipitation in the abdominal space if sodium sulfathiazole was injected. After sodium sulfapyridine and sulfamethylthiazole small amounts of drug-precipitates were found usually on the liver surface in almost all animals; even when sacrificed after 3 days. This finding is in agreement with the different solubilities of the free compounds and suggests that both sulfapyridine and sulfamethylthiazole are somewhat more toxic than indicated in the table. The small difference in toxicity for mice between sulfapyridine and sulfamethylthiazole as reported by Long and co-workers[2] using subcutaneous injection, can be explained in the light of our own experiments with intraperitoneal injection: in part as a consequence of unfavorable conditions of absorption from the subcutis of relatively insoluble substances, but chiefly from the fact that mice do not acetylate appreciably, and thus do not detoxify sulfapyridine to as great an extent as rats.

The early death after injection of large amounts of sodium sulfapyridine reported by van Dyke and co-workers,[3] and confirmed in our experiments (Table I), seems to be due to the high alkalinity of this compound. Smaller amounts do not exhaust the buffer capacity of the body and, therefore, will not influence the toxicity of the free compound considerably. If, however, the dose is excessive so that neutralization by the available acid reserve is not complete, the toxic picture may be complicated by symptoms of alkalosis. According to the buffer range these symptoms would be expected to be more pronounced after the administration of sodium sulfapyridine than after injection of the sodium salts of the thiazole derivatives.

The assumption is supported by data derived from potentiometric measurements of the pH in peritoneal exudate of rats dying only a few hours after intraperitoneal injection of sodium sulfapyridine and of the pH in blood of rats killed shortly after the intraperitoneal administration of the compound. In both cases definite deviation to the alkaline side could be demonstrated (7.6-7.8 for blood, 7.7 to 8.0 for peritoneal exudate).

The above contention is further strengthened by the fact that intraperitoneal injection of sulfapyridine as a fine suspension even in amounts equivalent to 5 times the absolute lethal dose of sodium sulfapyridine caused but slight depression and none of the symptoms appearing a few minutes after administration of the sodium salt, whereas sulfathiazole in a dose equimolar to the absolute lethal dose of its sodium salt killed 50% of the animals. The toxic picture caused by sulfathiazole was not unlike that seen after injection of its sodium salt.

The results of van Dyke and associates with oral administration[3] are similar to our findings. They report sodium sulfathiazole to have about 65% of the toxicity of sodium sulfapyridine, whereas upon feeding large amounts of the free compounds, sulfathiazole appeared to be the more toxic.

It is obvious from the data presented that toxicity determinations with the sodium salts of sulfanilamide derivatives having different alkalinities will not give a true picture of the toxicity of the free compounds. At present, therefore, the free compounds injected intraperitoneally as suspensions are being compared.

Conclusions. It has been shown that the acute toxicity of sodium sulfamethylthiazole in rats, as measured by intraperitoneal injection, is almost twice as high as that of sodium sulfathiazole, which in turn is slightly greater than the toxicity of sodium sulfapyridine.

The pH and buffer capacities of solutions of the sodium salts, and differences in solubility of the free compounds as factors influencing the toxicity, are discussed.

11568

Retention of Water by Pituitary (Posterior Lobe) Extract in Winter Frogs.

ELDON M. BOYD, M. E. CLARKE, E. L. CLARKE AND A. L. SEGAL.
From the Department of Pharmacology, Queen's University, Kingston, Canada.

The present report deals with further data bearing upon the mechanism of seasonal variation in the Brunn Reaction. The Brunn Reaction consists of an uptake of water by frogs when injected with suitable doses of pituitary (posterior lobe) extract and it is quantitatively greater in the summer than in the winter.[1] Certain aspects of the seasonal variation have been investigated in this laboratory. The reported increase in the winter of the number of acidophilic cells in the buccal lobe of the frog hypophysis lead us to inject various anterior pituitary and anterior pituitary-like principles along with extract of the posterior lobe but these additions did not depress the

---

[1] Boyd, E. M., Mack, E. G., and Smith, A. E., *Am. J. Physiol.*, 1939, **127**, 328.

Brunn Reaction in summer frogs.[2] A corresponding seasonal variation does not occur in albino rats given water by mouth with and without posthypophyseal extract.[3]

Boyd and Whyte[4] presented evidence which suggested that the Brunn Reaction is due not to a stimulation of water intake, which is the explanation usually offered, but to an inhibition of the normal loss of water by frogs immersed in water and to a consequent retention of water which is taken up over the time interval of the Brunn Reaction. The experiments which lead to this hypothesis consisted of measurement of the effect of pituitary (posterior lobe) extract on the loss of normal body water and of water injected into frogs which are taken out of water at the beginning of the period of measurement, in contrast to the procedure in the Brunn Reaction in which they are left in water throughout. Under these conditions, doses of pituitary (posterior lobe) extract corresponding to those used in the Brunn Reaction caused a retention of normal body water and of water injected into frogs. Further experiments demonstrated that dehydrated frogs are capable of taking up, and 'hydrated' frogs are capable of losing per hour amounts of water corresponding to those taken up and retained in the Brunn Reaction. Correlating these observations, the above explanation of the Brunn Reaction was offered.

The experiments of Boyd and Whyte[4] were performed in the summer months of 1938 and were confirmed in this laboratory by Mr. J. E. Brown in the summer of 1939. In January of 1940, several separate extended studies on what we may call the Boyd-Whyte Reaction were undertaken. None of us were able to obtain a normal Boyd-Whyte Reaction in spite of many repetitions. It occurred to us that this phenomenon might be due to a seasonal variation since experiments on the Boyd-Whyte Reaction had not been previously made during the winter in this laboratory. Repeating our experiments each week, we found that the Boyd-Whyte Reaction became established again rather suddenly toward the end of April and first of May at about the same time that the Brunn Reaction reaches its summer proportions.[1] We wish to report these changes in the Boyd-Whyte Reaction which accompany seasonal variation in the Brunn Reaction.

Method. Leopard frogs *(R. pipiens)* were acclimatized to room temperature in water for a period of 18 hours or longer in the months of January, February and March of 1940. At the beginning of a period of measurement, they were removed from water, urine expressed the skin thoroughly dried and body weight determined to the nearest 0.1 g on a trip scale balance. Half of the animals in each group received into the dorsal lymph sac an injection of a given dose of Pituitrin Surgical (generously supplied by Dr. E. A. Sharp of Parke, Davis and Company which company also provided a grant that defrayed part of the expenses of this investigation). The remaining half of the animals in each group served as controls and received by injection an equivalent volume of the vehicle of Pituitrin Surgical. Both groups were then re-weighed at hourly intervals for 5 to 6 hours. This procedure was repeated until there was a total of at least 50 frogs to a group. Percentage changes in body weight were then calculated and plotted against time.

---

[2] Boyd, E. M., and Gibson, J. D., PROC. SOC. EXP. BIOL. AND MED., 1940, **43**, 572.

[3] Boyd, E. M., and Garand, N. D., *Am. J. Physiol.*, 1940, **130**, 403.

[4] Boyd, E. M., and Whyte, D. W., *Am. J. Physiol.*, 1939, **125**, 415.

Normal Body Water. When frogs were taken out of water at room temperature in the winter months they were found to lose water at the rate of approximately 1% per hour. When pituitary (posterior lobe) extract was injected into these frogs in doses of 0.1 to 0.5 international units per 10 g body weight (corresponding to doses used in the Boyd-Whyte Reaction), the animals continued to lose weight at exactly the same rate. In other words, the Boyd-Whyte Reaction could not be demonstrated at this season of the year. At the same season of the year, the Brunn Reaction had decreased to about one-half its summer maximum but did not entirely disappear as did the Boyd-Whyte Reaction.

Dosage of Extract. It was considered possible that frogs in the winter months had merely lost their sensitivity to pituitary (posterior lobe) extract and the above experiments were repeated using a wide range of doses from 10^{-15} to 20 international units per 10 g of frog. For doses above 5 units, we could not use Pituitrin Surgical because the presence of chlorbutol in the vehicle rendered the extract lethal to frogs in such large amounts. We accordingly prepared our own extract from standard pituitary powder containing 2 international units per milligram by the method outlined in the British Pharmacopoeia, 1932, and this extract was used in the experiments with high doses. All of the results were entirely negative, even with the high doses. We had to conclude that the loss of normal body water of frogs could not be prevented in winter by doses of pituitary (posterior lobe) extract up to 20 international units per 10 g of frog (equivalent to 15 liters of Pituitrin Obstetrical injected into an average woman).

Administered Water. Boyd and Whyte[4] also demonstrated that when frogs in the summer are injected with 10% of their weight of distilled water into the dorsal lymph sac, a simultaneous intramuscular injection of Pituitrin Surgical in doses of 0.1 to 0.5 international units per 10 g will cause retention of the injected water as well as of normal body water. Repeating this experiment in the winter, we were able to show that pituitary (posterior lobe) extract is still capable of causing a retention of some of the injected water although it has lost the power to cause a retention of normal body water. In experiments on some 300 frogs, the mean retention of injected water was 40% over a period of 3 hours. In over 90% of the frogs the injected water was retained by the neurohypophyseal extract. The percentage retention of injected water varied considerably but on the average was below that in the summer, at which time up to 100% retention was recorded in the first 3 hours.

These results demonstrate that pituitary (posterior lobe) extract has an effect on administered water different from that on normal body water because at a time when the extract was not capable of causing a retention of normal body water it was capable of causing a retention of administered water. The results of the present study of the Boyd-Whyte Reaction and those of Boyd, Mack and Smith[1] on the Brunn Reaction combine to demonstrate a truly remarkable seasonal variation in the water metabolism of frogs.

Conclusion. The depression of the Brunn Reaction in winter months reported by Boyd, Mack and Smith[1] was found accompanied by complete failure of pituitary (posterior lobe) extract to inhibit loss of normal body water in frogs. On the other hand, the loss of water injected into frogs was inhibited by pituitary (posterior lobe) extract in the winter though to an average extent less than in the summer.

11569 P

Modifications in Development of the Sand Dollar by NaCNS.

Olin Rulon. (Introduced by C. M. Child.)

From the Hopkins Marine Station, Pacific Grove, and the Department of Biology, Wayne University, Detroit.

Experiments conducted at the Hopkins Marine Station, Pacific Grove, California, during the summer of 1938 on the unfertilized and fertilized eggs of *Dendraster excentricus* have given certain interesting modifications in the early developmental patterns of this form.

Unfertilized eggs treated for 12 hours with a solution of NaCNS (20 cc .54 M NaCNS plus 100 cc Ca-free sea water) showed a retardation in cleavage rate when returned to normal sea water and fertilized. The developing larvae ranged from forms showing considerable inhibition (increased mesenchyme, shortened anal arms, undifferentiated oral lobe) to larvae (approximately 35%) which consisted entirely of ectoderm and ectodermal structures. These ectodermized forms were modifications in the same direction as the 'animalized' forms produced in the sea urchin by Lindahl[1] and Hörstadius[2] with NaCNS treatment and experimental isolation of animal blastomeres. Neither mesenchyme, pigment nor gut was present. With higher concentrations or exposure periods the number of ectodermized forms was decreased and the number of inhibited forms increased. When NaCNS was used in normal sea water only inhibited larvae resulted. Ca-free sea water alone had little effect.

Fertilized eggs treated with NaCNS (20-30 cc .54 M NaCNS plus 100 cc normal sea water) for 12-24 hours appeared as thick-walled radially symmetrical blastulae usually containing a surplus of mesenchyme and showing a loss of cells from the vegetal region. When these forms were returned to normal sea water as many as 95% developed into exogastrulae with large evaginated entoderms, often filled with mesenchyme, and with ectoderm reduced to a small pigmented knob. Such forms were almost entirely entodermized and have been obtained by other workers (Herbst,[3] Child,[4] Hörstadius,[2] etc.) with various methods in this and other forms. If the treatment was less severe the ectoderm was usually quite large and underwent considerable differentiation. Fertilized eggs treated with Ca-free NaCNS solutions gave similar results if the period of exposure was short. If the treatment was longer than 8 hours the cells of the blastulae tended to separate. This was no doubt due to the calcium deficiency of the solution.

Contrary to Lindahl's view this work clearly indicates that the ectodermized forms produced by NaCNS treatment before fertilization result from recovery following a differential inhibition. The evidence lies in the following facts. *1. With optimum concentration and exposure period for ectodermization the maximum is only 35% and many larvae are not ectodermized at all but differentially inhibited, that is modified in the opposite direction. 2. With increase*

---

[1] Lindahl, P. E., *Acta zool.*, 1936, **17**, 179.

[2] Hörstadius, S., *Pubbl. Staz. zool. Napoli*, 1935, **14**, 251; *Biol. Rev.*, 1939, **14**, 132.

[3] Herbst, C., *Mitt. zool. Sta. Neapel.*, 1893, **11**, 136.

[4] Child, C. M., *Physiol. Zool.*, 1940, **13**, 4.

in concentration of agent or of exposure period percentages of ectodermized forms decrease, those of differentially inhibited forms increase. This is what would be expected from an inhibitory agent. The fact that cleavage and development are retarded further indicates the inhibitory action of NaCNS. Ectodermized forms result only when the treatment is sufficiently light to permit recovery of some individuals yet strong enough to affect the primary pattern. The exposure of unfertilized eggs to NaCNS in Ca-free sea water appears to result in a differential inhibition of the normal axiate pattern. With recovery on return to normal sea water and fertilization the basal levels of the egg seem to be freed to some extent from apical influence and develop in the same, or almost the same, direction as the originally higher levels. In other words the whole egg now develops as the animal portion with an increased scale of organization and a diminution or absence of entoderm and mesenchyme. When the early developmental stages (*i.e.*, first 12 hours after fertilization) are spent in solutions of NaCNS the resulting entodermization seems to follow from the depression of the more basal levels of prospective ectoderm to the physiological level of prospective entoderm. Such entodermized ectoderm may be considerably increased in size as a result of recovery on return to normal sea water. The increase in what appears to be mesenchyme results largely or wholly from dissociation and passage into the blastocoel of cells from the original entoderm. Child[4] has given a similar interpretation for entodermized larvae resulting from treatment with lithium.

11570

Variations in Arginase Activity in Livers of White Rats, Caused by Fasting.

HOWARD D. LIGHTBODY AND ABRAM KLEINMAN. (Introduced by D. B. Jones.)

From the Division of Pharmacology, Food and Drug Administration, U. S. Department of Agriculture, Washington, D. C.

Baldwin[1] has indicated that the liver arginase of some animal species is dependent upon the nutritional condition. Takehara[2] determined arginase in the livers and the kidneys of 4 male dogs after fasts of 7 to 23 days. He concluded that "the value for arginase in liver and kidney is markedly increased by fasting." The writers have statistically analyzed the results of Takehara and find that the differences between the means are not significant.

---

[1] Baldwin, E., *Biol. Rev.*, 1936, **11**, 247.

[2] Takehara, H. J., *Biochem.* (Japan), 1938, **28**, 309.

The object of this investigation was to study the variations, caused by fasting, in the quantities of arginase present in the livers of white rats.

Procedure. Methods of care of the animals, the collection of samples, and the estimation of the arginase activity have been described elsewhere.[3]

Five groups of rats were studied and all data concerning them are presented in Table I. The animals, with the exception of those in the large group of males fasted for 5 days, were between 92 and 104 days old when sacrificed. The age range of the large group was 102 to 136 days. This wider age spread was necessary in order to obtain the desired range in body weights.

The different periods of fast were selected because of the relationship that has been shown between the quantity of liver arginase and the quantity of protein catabolized.[4] The 2-day fast period was selected because it has been shown by Addis, Poo and Lew[5] that a 2-day fast markedly reduces the protein content of the livers of white rats. The 8-day fast for one group of males and one of females was selected because it represents approximately the maximum survival time of rats under the conditions used in these experiments. A fast of 5 days for a group of males was selected since preliminary experiments had shown that it represents a stage of fasting previous to the time of onset of the "premortal rise" in nitrogen excretion. The number of animals in this group was large and statistical methods were used as an aid in interpretation of the data. Females were selected for the fifth group because it has been shown[3, 4] that livers of female rats at ages of about 100 days contain less arginase than do those of male rats of similar age. The 4-day fast was selected for this group since we were interested in the adjustment in the concentration of liver arginase by females during a time when the store of protein in the liver has been largely depleted, as indicated by the work of Addis, Poo and Lew,[5] and while there yet remains a considerable quantity of body fat.

Discussion. Adaptations in the quantity of liver arginase to the quantity of catabolized protein nitrogen produced by changes in protein consumption are reflected in a change in the size of the liver, which appears first, and in the concentration of the enzyme in the liver, which follows.[4] Evidence of a similar twofold adjustment may be seen in the results of the experiments reported in this paper. The concentrations of liver arginase were somewhat increased in male rats fasted for 2 days, but the size of the organ decreased about 28%, resulting in a marked decrease in the total quantity of the enzyme per unit of body weight. Females fasted for 4 days reacted in a similar manner, but failed to show an increase in the concentration, the total arginase per unit of body weight being reduced even more than in male rats which were fasted for 2 days. Adjustments by the sexes to prolonged fasts of 8 days were likewise made by somewhat different means. For example, the quantity of liver tissue per 100 g of body weight of male rats was found to have further decreased and the amount of enzyme per unit of weight of liver markedly increased. The sizes of the livers of the female rats were maintained from the fourth to the eighth day of fasting and the adjustment indicated solely by increase in the enzyme concentration. The quantity of liver arginase per unit of body weight after a fast of 8 days was found to be equal to, or exceed that in livers of rats fed a diet containing 25% milk proteins, though in neither sex was the concentration as great as that found after feeding a 75% protein mixture.[4]

---

[3] Lightbody, H. D., *J. Biol. Chem.*, 1938, **124**, 169.

[4] Lightbody, H. D., and Kleinman, A., *J. Biol. Chem.*, 1939, **129**, 71.

[5] Addis, T., Poo, J., and Lew, W., *J. Biol. Chem.*, 1936, **115**, 117.

The results obtained after fasting male rats for 5 days (Fig. 1) indicate that a definite relationship exists between body weight (used here as an index of reserve nonprotein energy materials) and the amount of liver arginase per unit of body weight. The smaller animals were found to contain quantities equal to or greater than the quantities for those animals of comparable ages permitted food near optimum in protein content.[4] In some cases the amount of enzyme was found to approach that found after fasts of 8 days. In contrast, heavy animals have smaller quantities of the enzyme per unit of body

TABLE I.
Influence of Fasting on the Arginase Content of Liver.
(With the exceptions noted, the values are group means.)

Sex	No. of animals	Duration of fast, days	Body wt loss, %	Livers			
				Solids, %	Dry, per 100 g rat, g	*Arginase units	
						Per mg dry liver	Per 100 g rat $\times 10^{-3}$
M	15	2	11.93	33.0±0.3	0.83±0.01	253.1± 6.3	211.1±6.7
M	18	8	32.05	33.0±0.3	0.75±0.02	337.8± 9.8	252.3±6.4
F	15	8	30.58	32.1±0.3	0.83±0.02	283.7±12.5	234.9±9.8
M	26	5	16.11-22.50†	30.2-35.9†	0.65-1.10†	166.3-317.2†	120.8-249.5†
F	14	4	18.89	32.9±0.2	0.83±0.02	197.7± 8.3	164.7±9.5

*The arginase unit is that quantity of the enzyme which will liberate urea equivalent to one micromole ($M \times 10^{-6}$) of carbon dioxide under the experimental conditions used.

†Range in values.

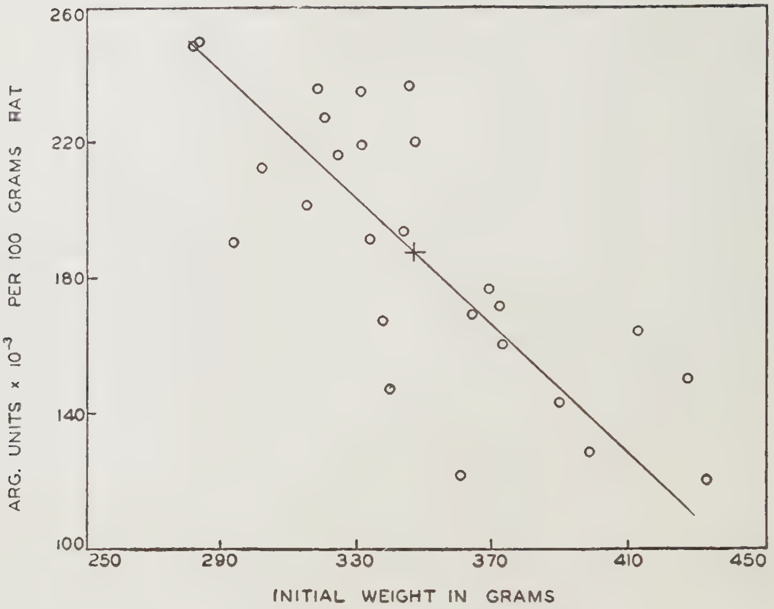


FIG. 1.

The regression line, as calculated by the method of least squares, from the results obtained by fasting male rats for 5 days.

weight, after a fast of 5 days, than have any yet examined during these studies.

If the quantity of liver arginase per unit of body weight is used as an index of the demands placed upon the enzyme system concerned with protein catabolism, the response of male rats to prolonged fast may be divided into 3 periods. The first of these is characterized by decrease in total liver arginase per unit of body weight reflected by a decrease in the quantity of liver tissue while maintaining the concentration. The second period, one of variable duration, is indicated by further decreases in the size of the liver and of the quantity of enzyme per unit weight of liver tissue. Finally there ensues a time after prolonged fast when the concentration of the enzyme in the liver is markedly increased, and the quantity of the enzyme present per unit of body weight may become equal to or greater than that found in animals fed food near optimum in protein content.[4] The adjustments in female rats when fasted 8 days seem to have been effected in manners similar to those in males.

Hutchinson and Morris[6] suggest that "either the caloric requirements immediately following starvation may be higher than normal requirements, resulting in the deamination of amino acids for energy purposes, or the ability of the animal to retain absorbed N may be somewhat impaired following a prolonged period of undernutrition." In view of our findings we feel that the second possibility is the more likely explanation. The ability to utilize amino acids (as indicated by the ability to dispose of the waste nitrogen as urea) upon refeeding would appear to be somewhat dependent on the duration of the fast. If the fast were terminated and protein feeding resumed during a period when chiefly nonprotein materials were being used as the energy sources and at a time when the metabolic enzyme systems were adjusted to the utilization of carbohydrates or fats, the expected result would be poor utilization of the ingested protein, because of a deficiency of enzymes concerned with protein metabolism, and be reflected in nitrogen excretion characterized by low urea and high amino acid fractions. Efficient protein utilization at the new level of intake must be expected to await readjustment of the enzyme systems concerned in protein metabolism.

---

[6] Hutchinson, J. C. D., and Morris, S., *Biochem. J.*, 1936, **30**, 1695.

Conclusions. Rats respond to short fasts (2 days) without change in arginase concentration in the liver tissue, and adaptations to changes in metabolism are reflected in decreases in the size of the liver. If fasted until near the moribund condition there is increased arginase concentration in the livers of both sexes, with loss of sex differentiation. Adjustment of the quantities of this enzyme by male rats when fasted 8 days is reflected by change in the size of the liver and by increase in its concentration of the enzyme. Adjustments to long fasts by female rats is accompanied by increase in enzyme concentration and after 4 days of fast the quantities of liver tissue are not further decreased. As shown in Fig. 1 fasts of intermediate duration give variable results in the concentration of the enzyme in the livers of male rats. Such variations may be correlated with the type of body materials, carbohydrates and fats (non-nitrogenous) or proteins (nitrogenous), that serve as primary energy sources at the time of sacrifice. Expressed as quantity of enzyme per unit of body weight, arginase activity changes during the adjustment to the expected utilization of proteins as energy sources.

11571 P

An Electron Microscope Study of Calcium and Magnesium in Smooth Muscle.*

Gordon H. Scott.

From the Department of Anatomy, Washington University School of Medicine, St. Louis, Mo.

Recent investigations of electrolytes in Thyone muscle by Steinbach[1] have led him to the conclusion that Ca is present in cells in lower concentration than that of the surrounding medium. Since these results are at variance with our own in a number of respects we have studied again the localization of Ca and Mg in mammalian and frog smooth muscle by the electron microscope as described by Scott and Packer.[2] Naturally, the results obtained cannot be compared directly with those of Steinbach as the same form was not used. Furthermore, our interest lay in determining the localization in smooth muscle cells which had been preserved as nearly as possible in their normal physiological state.

Thin strips of smooth muscle were taken from the duodenum and stomach wall of cats and frogs. These pieces were frozen in liquid air while still actively contracting from the mechanical stimulation of the manipulation. The frozen tissues were dehydrated *in vacuo* at –63°C and infiltrated in water-free paraffin without breaking the vacuum. This procedure precludes any salt shift during dehydration and the addition of water from outside sources at any time and thus maintains the spatial relations of the element in question. Thin sections (8μ) of muscle were prepared and examined by heating on a surfaced cathode in the electron microscope.

The technic of preparation of tissues for examination and the method of localizing Ca and Mg are believed to preserve the exact topical cellular relationships. Unfortunately, it is impossible by the method used to differentiate Ca and Mg, since both activate the cathode surface. Quantities of these elements in less than 1×10^{-12} g are easily detected. In no instance was either Ca or Mg found outside the cell wall. Smooth muscle cells of the frog duodenum show a dense concentration of Ca and Mg in the cell. Occasionally delicate threads of mineral traverse the tissue spaces to connect adjacent cells. When stained sections are examined one finds that there are protoplasmic extensions connecting the cells and that these filaments are surrounded by clearly demonstrable cell walls. In mammalian smooth muscle this particular situation does not exist insofar as could be ascertained. The tissue spaces are extremely small and difficult to locate. One of the striking features in mammalian muscle is the clearness with which cell boundaries can be determined. Yet Ca and Mg were by no means, either in frog or mammalian smooth muscle, limited to the periphery of the cell.

---

* Aided by a grant from the Rockefeller Foundation.

[1] Steinbach, *J. Cell. and Comp. Physiol.*, 1940, **15**, 1.

[2] Scott and Packer, *Anat. Rec.*, 1939, **74**, 17, 31.

These findings do not imply that there is no Ca or Mg in the tissue spaces. The conclusion is apparent, however, that under conditions of normal existence the Ca and Mg concentration is many times greater within the smooth muscle cells of the forms studied than in their surrounding tissue fluid environment.

11572 P

Protective Effect of Sulfamethylthiazol* on Experimental *Salmonella enteritidis* Infection in Mice.

R. D. MUIR, VIRGINIA SHAMLEFFER AND L. R. JONES.

From the Department of Bacteriology, St. Louis University School of Medicine, St. Louis, Mo.

In a previous report[1] the effect of sulfamethylthiazol upon the course of experimental staphylococcus infection in mice was presented. In further studies of the protective effect of this drug against experimental infections in mice a series of experiments have been made wherein the infecting organism was a strain of *Salmonella enteritidis*.† The organism had been previously recovered as the etiological agent in an epizootic disease among the mice of our colony.

Preliminary studies regarding the pathogenicity of this organism for mice of a selected strain, known to be free from Salmonella organisms, indicated that animals succumbed to the organism as a result of a progressive infection rather than to the presence of pre-formed toxin factors present in the inoculum. A dose of one million cells, injected intraperitoneally, brought about the death of a majority of the animals within 7 days. A similar inoculation of from 75 to 100 million organisms consistently killed mice within 24 hours. Attempts to produce infection *per os* were unsuccessful.

---

* Supplied through the courtesy of the Department of Medical Research of the Winthrop Chemical Co., New York.

[1] Carroll, G., Kappel, L., Jones, L., Gallagher, F. W., and DiRocco, F. W., *South. Med. J.*, 1940, **33**, 83.

† We are indebted to Dr. P. R. Edwards for determining the serological character of this organism.

Methods. A number of experiments were carried out in order to ascertain the possible effect of sulfamethylthiazol on the course of experimental infection in mice. The drug was administered on a dosage basis of 4 g per kilo of body weight per day, half of the required amount being given in the morning and the remainder 10 to 12 hours later. Because of its insolubility, the drug was suspended in milk so that the desired dose was contained in 0.2 cc of the suspension, and this amount was placed directly into the stomach of the animal by means of a specially prepared 18-gauge hypodermic needle having a solid blunt end and an opening along the side. In control experiments it was found that milk *per se* was without effect in altering the survival time of infected mice. Control animals were always maintained which were infected but not medicated.

Mice were experimentally infected by the intraperitoneal injection of 0.1 cc of a cell suspension which was derived from 24-hour broth cultures of the organism. The numbers of viable organisms in these suspensions were determined by plate counts.

Results. The data incorporated in Table I indicate a significant protective effect of sulfamethylthiazol. This was further confirmed in another experiment involving the administration of 90 million *Salmonella enteritidis* cells per mouse, the average survival time being 14 hours for the control group and 92 hours for the medicated group. In a third experimental series wherein 87 million organisms were injected, the survival times were 15.4 hours and 95.6 hours respectively.

TABLE I.
Protective Effect of Sulfamethylthiazol in Mice Infected with *Salmonella enteritidis.*
Hours of Survival Following Injection of 85 Million Cells.

Control animals (non-medicated)	Medicated animals
13.5	13.5
13.5	20.5
14.5	38.5
14.5	49.5
16.5	74.5
16.5	92.5
16.5	115.5
18.5	117.5
18.5	181.5
20.0	181.5
Avg survival time 16.25 hr	88.5 hr

In the above experiments, the drug was first administered 2 hours prior to the injection of the organisms. In another group of experiments we compared the effectiveness of sulfamethylthiazol in protecting mice against experimental Salmonella infections when the initial administration of the drug was (1) at the time of injection of the organisms, and (2) 14 hours previous to the injection of the organisms. The non-medicated control animals survived, on the average, 15 hours. The animals first medicated at the time of the injection of the organisms had an average survival time of 96 hours, thus living more than 6 times as long as the untreated animals. Those which were first medicated 14 hours before and again 1 hour before injection and subsequently medicated according to the usual scheme, survived an average of 110 hours, or more than 7 times as long as the controls. Thus it becomes apparent that pre-medication, which conceivably leads to the development of a certain concentration of the drug in the blood at the time of injection, increases the protection afforded the animals.

The adequacy of dosage was partially investigated by administering to a series of experimental animals only one-half (2 g per kg per day) of the usual amount of drug, starting the medication program at the time of injection. These mice showed an average survival time of 51 hours, or more than 3 times that of the control group. Accordingly, it would seem that when the posology scheme is quantitatively reduced by one-half there is a significant decrease in the protective effect of the drug.

Conclusions. Sulfamethylthiazol apparently exerts a definite protective effect in mice which have been experimentally infected with a virulent strain of *Salmonella enteritidis*. This protective effect is manifested only by an increase in average survival time of medicated animals. Eventually all of the medicated animals succumb to the *Salmonella enteritidis* infection.

11573

Androgens and Uterine Weight in the Immature Rat.*

R. R. Greene and S. C. Harris. (Introduced by A. C. Ivy.)

From the Department of Physiology and Pharmacology, Northwestern University Medical School, Chicago.

Astwood has demonstrated that 6 hours after the administration of estrogens to the 21-day-old rat, there is an increase in uterine weight.[1] This weight increase is largely due to increased hydration (edema) of the uterus. Pincus and Graubard have essentially confirmed these findings.[2] Zuckerman has stated that androgens also cause increased hydration of the uterus in the monkey.[3] Astwood in a brief report has stated that androgens will cause a uterine weight increase in rats similar to that caused by estrogens.[4]

The Astwood technic as originally proposed for estrogen assays is a very rapid and convenient one. Reports have appeared in which it has been applied to urinary estrogen assays.[5] Inasmuch as androgens are known to be present in the urines of normal, pregnant or castrate women, it seems of interest to report our findings as to the uterine weight-increasing effects of various androgens.

Findings. All animals were given the androgen subcutaneously in 0.1 cc of peanut oil and were killed 6 hours after the treatment. Peanut oil has previously been tested in this laboratory and found to contain neither estrogen nor androgen. The cervico-vaginal junctions were cut, the uteri carefully removed and immediately weighed on a Roller-Smith balance sensitive to 0.02 mg. The tissues were preserved in Bouin's fluid and representative uteri were examined microscopically. Detailed weight-findings are presented in tabular form (Table I).

The animals of Groups 1 and 2 were 21 days old. Group 1 served as controls (A). As can be noted in the table, 0.5 mg testosterone (Group 2) caused a statistically significant increase in uterine weight, 22.8% over that of the controls.

The 21-day-old animals used in these groups showed a great deal

---

* Supported in part by a grant from the Josiah Macy, Jr., Foundation. The testosterone and dehydroandrosterone were furnished by Ciba Pharmaceutical Products, Inc.

[1] Astwood, E. B., *Am. J. Physiol.*, 1939, **126**, 162.

[2] Pincus, G., and Graubard, M., *Endocrinology*, 1940, **26**, 684.

[3] Zuckerman, S., *J. Endocrin.*, 1939, **1**, 147.

[4] Astwood, E. B., *Proc. Am. Physiol. Soc.*, 1940, p. 6.

[5] Pincus, G., and Graubard, M., *Endocrinology*, 1940, **26**, 427.

TABLE I.
The Effect of Androgens on Uterine Weight.

Group	Treatment		No. of animals	Body wt, g	Uterine wt, mg			Obtained difference	C.R.	% increase in uterine wt
	Substance	Mg			Mean	Range	σ			
1	Control A	—	20	28.4	21.74	15.66-29.8	0.273	—	—	—
2	Testosterone	0.5	20	28.6	26.81	19.4 -36.92	0.949	5.07	*3.94	22.8
3	Control B	—	20	28.3	21.05	15.74-27.04	0.182	—	—	—
4	Testosterone	0.1	20	28.02	21.78	16.52-27.56	0.249	0.73	2.94	3.4
5	Dehydroand.	1.5	20	28.2	26.29	17.90-37.92	1.14	5.24	*4.09	24.9
6	”	0.5	23	28.47	24.56	17.58-34.15	0.265	3.51	*3.44	16.6

σ (Standard Error of the Mean) $= \sqrt{\frac{\Sigma\delta^2}{n(n-1)}}$ C.R. = Critical Ratio $= \frac{\text{obtained difference}}{\sqrt{(\sigma_{m_1})^2 + (\sigma_{m_2})^2}}$

*Statistically significant increase in uterine weight over that of controls (C.R. > 3.0).

of variation in body weight (range 22 to 36 g). The uterine weights of the controls ranged from 15.66 to 29.8 mg with a standard error of the mean of 0.273 mg. The range and standard error were even larger in the testosterone-treated group (range: 19.4 to 36.92 mg and σ of 0.949).

An endeavor was made to keep the body weights less variable in the subsequent control and experimental groups (Groups 3 through 6) in the hope that the uterine weights would show less variability. Accordingly, the animals in these groups were limited to those of a body weight of 24 to 32 g, but varied in age from 19 to 23 days.

The uteri of the second control (B) group showed only a slightly smaller range but the intra-group variability was less (σ 0.182). In the remaining treated groups the range of the uterine weights was unaffected by more rigidly limiting the body weight, but with the exception of Group 5, the intra-group variability did seem to be decreased.

The animals of Group 4 were given 0.1 mg testosterone. The uteri of these animals were 3.4% larger than those of the controls (Group 3). This increase in uterine weight, however, fell slightly short of being statistically significant (C.R. was 2.94 instead of being 3.00 or over).

The animals of Group 5 were given 1.5 mg of the urinary androgen, dehydroandrosterone. The uteri of these animals were significantly increased in weight (24.9%). The animals of Group 6 were given 0.5 mg dehydroandrosterone. The uteri of these animals were also significantly larger (16.6%) than those of the controls.

Histological examination of the uteri from the treated animals revealed an appreciable edema of the endometrial stroma. This edema was either most noticeable in, or was limited to, the portion of the endometrial stroma closest to the muscular layer.

The unit as proposed by Astwood for use in estrogenic assay is the amount of estrogen that will cause a 34% increase in uterine weight. This weight increase is produced in 6 hours by 0.025 γ of estradiol or 0.3 γ of estrone. Our data are too limited to make any accurate comparison of the potency of androgens or estrogens in this regard. However, a 16.6% increase in uterine weight was obtained with the smallest dose of dehydroandrosterone used (0.5 mg). The *average* daily excretion of estrogen in the normal non-pregnant female has been calculated from the data of various authors[6, 7] as 190 to 464 international units, or equivalent in activity to 19.0 to 46.4 γ of estrone (63.3 to 154.66 "Astwood units"). The normal

---

[6] Smith, G. S., and Smith, O. W., *New England Med. J.*, 1936, **215**, 908.

[7] Palmer, A., PROC. SOC. EXP. BIOL. AND MED., 1937, **37**, 273.

female excretes 30 to 100 international units of androgen daily.[8, 9] The known androgens in female urine and their relative amounts are in a proportion of 1.3 mg androsterone to 0.2 mg dehydroandrosterone.[10] An international unit of androsterone is 0.1 mg and of dehydroandrosterone is 0.3 mg. With the maximum excretion of 100 international units this would be equivalent to approximately 8.7 mg androsterone and 4.0 mg of dehydroandrosterone. These urinary androgens, unless carefully separated from the estrogenic fraction, could then be sufficient to interfere seriously with the accuracy of an assay by the Astwood method in the non-pregnant female.

11574 P

Effect of Crude Peanut Oil Extracts of Brown Fat on Metabolism of White Rat.

WALTER E. HOOK. (Introduced by George F. Dick.)

From the Department of Medicine, University of Chicago.

This paper presents preliminary data on the effect of the intraperitoneal injection of crude peanut oil extracts of the brown fat of the woodchuck and the thirteen-lined ground squirrel on the heat production of the white rat.

The earlier literature on brown fat has been reviewed by Rasmussen.[1] Various theories as to function have been proposed, but there has been a paucity of chemical and biological studies. Brown fat has been noted in infants[2] and cretins.[3] More recent work has suggested a further relationship to metabolism.[4, 5, 6] Wendt[7] reported

---

[8] Koch, F. C., *Physiol. Rev.*, 1937, **17**, 153.

[9] Callow, N. H., Callow, R. K., Emmens, C. W., and Stroud, S. W., *J. Endocrin.*, 1939, **1**, 76.

[10] Callow, N. H., and Callow, R. K., *Biochem. J.*, 1939, **33**, 931.

[1] Rasmussen, A. T., *Endocrinology*, 1922, **6**, 760.

[2] Hatai, *Anat. Anzeiger*, 1902, **21**, 369; Batty Shaw, *J. Anat. and Physiol.*, 1902, **36**, 1; Bonnot, *J. Anat. and Physiol.*, 1908, **43**.

[3] Curling, T. B., *Med.-Chirurg. Transact. London*, 1850, **33**, 303; Hilton Fagge, *ibid.*, London, 1871, **54**, 154; Shattock, *Proc. Roy. Soc. Med.*, 2, Path. Sect., 1909, 252.

[4] Vignes, H., *Compt. Rend. des Sci. et Mem. de la Soc. Biol.*, 1913, **75**, 360.

[5] Felix, K., u. Eger, W., *Deutsches Arch. f. klin. Med.*, 1938, **182**, 41.

[6] Friedman u. Feinschmidt, *Charkow. Erg. d. Bio.*, 1932, **8**.

[7] Wendt, C. F., *Z. f. Phys. Chemie*, 1937, **249**, IV.

that injection of extracts of brown fat of hibernating hedgehogs into rats caused a lowering of the metabolism from 20-30% in 2-3 hours as measured by the Zeiss laboratory interferometer (method of preparation of extract not given). Control injections of olive oil did not cause this effect.

The experiments here reported were performed from April, 1939, to March, 1940. Hibernation was induced by first dehydrating the animals and then placing them in the cold (36°F-40°F) for 3 weeks to 3 months without food or water. The ground squirrels were killed by decapitation and the woodchucks by intracardiac air injection. Only axillary brown fat was used. The tissue was removed rapidly, immediately frozen in liquid nitrogen and later crushed in a tissue crusher.[8] The crushed tissue was then thoroughly ground in a mortar with peanut oil (approximately 5 cc of the latter per gram of tissue). After filtration of the supernatant fluid 1 cc of the filtrate was injected intraperitoneally into white, litter-mate rats. The rats were fed standard laboratory rations. The heat production was calculated from the oxygen consumption measured in an apparatus described elsewhere by Teague[9] and by Swann and Johnson.[10] Each rat was trained daily in the apparatus for 2 weeks before heat production studies were made and only well-trained rats were used.

The results are given in Table I. In the initial experiments during April and May, 1939, (Nos. 1, 2, 3) it was noted that injection of peanut oil alone elevated the heat production, whereas injection of crude peanut oil extracts of brown fat lowered it. Because of these results further experiments were performed during May and June, 1939. These failed to show any decrease in oxygen consumption within 3 hours after injection of the crude extract of brown fat. However, in several rats where the determination was made 2 days after injection (Nos. 4, 5, 6), there was, at that time, a marked depression of heat production. Because of this variability more detailed studies were made during July, 1939, (No. 7) which showed a definite fall in metabolism 1 and 2 days after injection. Controls did not show any changes. The injection of a similarly prepared crude extract into an hypophysectomized rat (No. 8) was followed by a sharp fall in heat production (35% in 3 hours) and the animal died 12 hours after injection (no gross findings at autopsy). The same crude extract caused a slight lowering of oxygen consumption in a normal rat (No. 9), whereas peanut oil alone caused a slight elevation (No. 10).

---

[8] Graeser, J. B., Ginsberg, J. E., and Friedemann, T. E., *J. Biol. Chem.*, 1934, **104**, 149.

[9] Teague, R. S., *Endocrinology*, 1939, **25**, 953.

[10] Swann, H. G., and Johnson, P. E., *Endocrinology*, 1939, **24**, 397.

TABLE I.

Comparison of Effects of Intraperitoneal Injection of Peanut Oil and of Crude Peanut Oil Extracts of Brown Fat of Woodchucks and Ground Squirrels on Heat Production of the White Rat.

Rat No.	Date 1939	Wt, g	Control	Injections 1 cc	Heat production Calories per square meter of body surface* per 24 hr							
					Hr after injection			Days after injection				
					1	2	3	1	2	3	5	6
1	4-20	186	715	P.O.[1]	700	1001	912		720			
	4-22	196	720	,,	790	841	892					
	5-2	196	732	B.F.[2]	470	470						
2	4-20	162	895	P.O.	910	1031	1175		901			
	4-22	170	901	,,		1131	1120					
	5-2	174	875	B.F.	579		761					
3	4-20	156	750	P.O.		1130	894		785			
	4-22	160	785	,,	845	826						
	5-2	164	800	B.F.	615	724						
4	6-18	252	820	B.F.		826			615			
	6-27	268	1080	,,		970			680			770
5	6-27	272	805	,,	809	785			666			785
6	6-18	250	799	,,	850				591		715	846
7	7-21	280	969	,,	870	972		695	575	790	790	815
8†	6-6	190	625	,,	560	442	405	Died 12 hr after inj.				
9	6-6	170	940	,,	870	897	940					
10	6-6	260	715	P.O.	765	832	841					
	1940											
11	3-16	225	1060	B.F.	725	800		846	755	970	980	
12	3-11	0.5 mg thyroxin intraperitoneally daily										
	3-16	190	1600	B.F.	1131	1058		1190	1690	1400		1410
	3-25	205	1410	P.O.	1230	1230		1715				
13	3-11	0.5 mg thyroxin intraperitoneally daily										
	3-18	191	1300	P.O.	1510	1510		1260				

*Surface area calculated from Rubner's formula $9.1\sqrt[3]{wt^2}$.

†Hypophysectomized rat.

[1] P.O.—Peanut oil.

[2] B.F.—Brown fat.

The effects of similarly prepared extracts of omental fat and kidney were studied in 4 experiments. In no instance was a lowering of the oxygen consumption noted after injection. During March, 1940, further determinations were again made and the effects of crude extracts were studied in 1 normal (No. 11) and 2 thyroidized rats (Nos. 12, 13).

Summary. It would appear that in these experiments the intraperitoneal injection of crude peanut oil extracts of the brown fat of the woodchuck and the thirteen-lined ground squirrel produced a lowering of the heat production of the white rat either at once or after 1 or 2 days (as measured by the oxygen consumption). Such effects, with one exception, were not produced by the injection of peanut oil alone.

11575

Protection of Guinea Pigs Against Mexican Typhus Virus by Vaccine from Infected Rat Lungs (Castaneda).

N. Paul Hudson.

From the Department of Medical Research, General Hospital, Mexico City, and the Department of Bacteriology, Ohio State University, Columbus.

In recent years much experimental work has been done on the production of a vaccine effective against typhus. The principal effort has been to devise a method that yields rickettsiae in sufficient quantity to be of practical application to the prevention of this disease. Since the use of infected lice as a source of rickettsiae, as developed by Weigl in 1930, the most promising methods have utilized peritoneal washings of infected white rats previously treated with benzol or irradiated with X-rays (Zinsser and Castaneda[1]); infected tissue cultures on agar slants (Zinsser, FitzPatrick and Wei[2]); material from the infected yolk sac of the developing chick (Cox, and Cox and Bell[3]); and a combination of the yolk sac and agar tissue culture methods (Zinsser, Plotz and Enders[4]). Castaneda has recently reported the production of an experimental typhus pneumonia in white rats[5] that yields large numbers of rickettsiae separable from infected lung tissue by differential centrifugation and available for use as a killed vaccine.[6]

---

[1] Zinsser, H., and Castaneda, M. R., *J. Exp. Med.*, 1930, **52**, 649; Proc. Soc. Exp. Biol. and Med., 1932, **29**, 840.

[2] Zinsser, H., FitzPatrick, F., and Wei, H., *J. Exp. Med.*, 1939, **69**, 179.

[3] Cox, H. R., *U. S. Pub. Health Rep.*, 1938, **53**, 2241; Cox, H. R., and Bell, E. J., *U. S. Pub. Health Rep.*, 1940, **55**, 110.

[4] Zinsser, H., Plotz, H., and Enders, J. F., *Science*, 1940, **91**, 51.

During a period of study in the typhus research laboratory of Dr. Castaneda, the writer had the opportunity to examine the effectiveness of the vaccine prepared from rat lungs in the protection of guinea pigs against typhus virus. This is a brief report of that work.

Methods. A Mexican strain ("L") of typhus was used both as the vaccine and as the test strain of live virus. The vaccine consisted of rickettsiae in pure suspension, killed by phenol, and numbering about one and a half billion per cc. It was injected subcutaneously into 10 male guinea pigs weighing from 400 to 570 g each. Two animals received 4 injections of 1 cc each; 2 were given 3 injections of 1 cc each; 2 were injected twice, 1 cc each; 2 once with 1 cc; and 2 once with 0.2 cc vaccine. The injections were made at intervals of 5 days and in such a way as to have all animals receive their last vaccine injections on the same day.

Eleven days later, all animals were given intraperitoneal inoculations of a test dose of virus, consisting of a suspension of scrapings from the tunica vaginalis of infected guinea pigs. Each vaccinated animal received in 5 cc sterile saline the equivalent of about 1/20 of the scrapings of one tunica; this is far above the minimal infecting dose. Four control guinea pigs, of approximately the same weight as those vaccinated, were injected with one-half the test dose of virus given the vaccinated guinea pigs.

The temperatures of all animals were taken daily, routinely late in the morning. The temperature of 40°C, and above, was taken arbitrarily to represent a fever. Degrees of scrotal reactions were recorded as from 1 to 4 plus. Daily observations were made for 17 days.

Results. The control (unvaccinated) guinea pigs had febrile readings scattered from the fourth to the eleventh day following inoculation. Each control animal also had a scrotal reaction, typical of the effect of an orchitic strain of Mexican typhus. These reactions appeared on the fifth and sixth days and lasted from 2 to 7 days. The degrees of reaction were graded from 1 to 4 plus.

One of the 10 vaccinated guinea pigs died of intercurrent infection (intestinal?) on the third day after inoculation with test virus. All other animals survived the test dose without fever or scrotal reaction. Three of these had temperatures of 40.0°, 40.2° and 40.3° on the fourteenth day after inoculation and after the febrile and acute scrotal reactions in the controls had subsided; these temperatures were not considered significant because of their late occurrence.

---

[5] Castaneda, M. R., *Am. J. Path.*, 1939, **15**, 467.

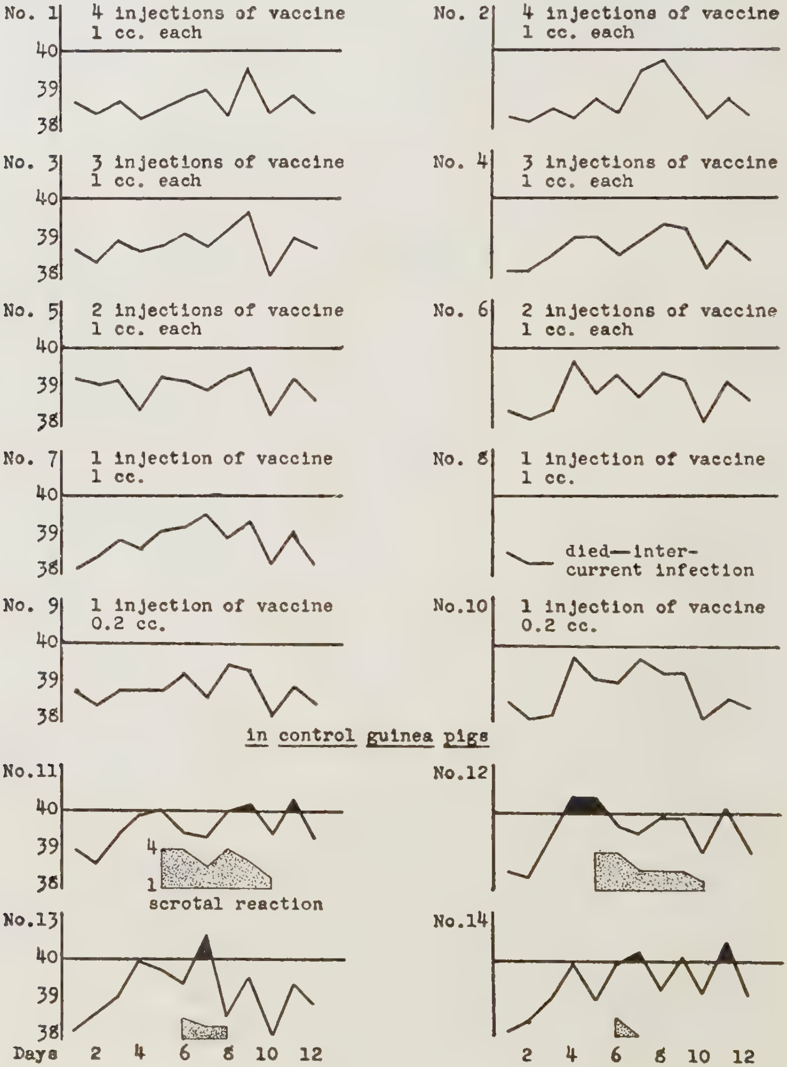
[6] Castaneda, M. R., prepared for publication.

Observations made on the animals during the 12-day period immediately following inoculation of the test dose of virus are presented in Chart 1.

CHART I.

Temperatures and scrotal reactions following test dose of typhus virus

in vaccinated guinea pigs



Summary. Typhus vaccine, consisting of killed rickettsiae from infected rat lungs prepared according to the method of Castaneda, protected guinea pigs against infective doses of virus injected intraperitoneally. Immunization was accomplished by the subcutaneous injection of the vaccine in as small a dose as 0.2 cc. The virus used in the preparation of the vaccine and for the test for immunity was the "L" strain of Mexican typhus.

11576

Inhibition of Tryptases by Heparin.*

ANTHONY J. GLAZKO AND JOHN H. FERGUSON.
From the Department of Materia Medica, University of Michigan, Ann Arbor.

It has been demonstrated by Fischer[1] that the isoelectric point of proteins shifts towards the acid side in the presence of heparin. He concluded that the acidic groups of heparin combined with the basic groups of proteins, forming salt-like compounds. Since trypsin attacks negatively charged proteins very readily,[2] it might be expected that the addition of heparin would facilitate proteolysis by making the protein behave as a stronger anion (at constant pH). However, trypsin is also a protein, and it may be that heparin can alter its activity by combining with it. The results of this paper indicate that heparin *decreases* the proteolytic activity of serum tryptase and crystalline trypsin.

Two methods were used to study the inhibitory action of heparin: (1) the increase in the non-protein nitrogen which resulted from the digestion of casein by trypsin was followed by means of micro-Kjeldahl analyses; and (2) the rate of inactivation of thrombin by trypsin or serum tryptase ("progressive" antithrombin) was estimated by assaying the coagulant activity of the residual thrombin.[3]

A. *Action of Heparin on Casein + Trypsin.* Various amounts of a heparin preparation† were added to 5 cc portions of a 3% casein solution, buffered to pH 8.2 with M/10 phosphate. The final volumes were adjusted to 7 cc, the mixtures warmed to 38°, and 1 cc

---

* This work was assisted by a grant from the Horace H. Rackham Fund.

[1] Fischer, A., *Biochem. Zeits.*, 1935, **278**, 133.

[2] Northrop, J. H., *Crystalline Enzymes* (New York), 1939.

[3] Glazko, A. J., and Ferguson, J. H., *J. Gen. Physiol.*, 1940, **24**, 169.

† Connaught Laboratories. The preparation assayed 110 units per mg.

(=0.5 mg) of a solution of crystalline trypsin‡ was added to each sample. One cc portions were withdrawn by pipette after specified time intervals and added to 4 cc portions of 10% trichloracetic acid. The mixtures were shaken and allowed to stand a half-hour before filtration. One cc of each filtrate was taken for analysis by the usual micro-Kjeldahl technic. The results of this procedure, corrected for the variation in NPN due to the heparin-N, are given in Table I. It is evident that the rate of proteolysis is decreased in the presence of heparin. In a similar experiment where crystalline papain was used in place of trypsin, no significant inhibition was observed.

TABLE I.
Inhibition by Heparin of Tryptic Digestion of Casein.

Heparin (mg/cc)	Time of incubation in min. 10′	20′	40′	80′	160′
	mg NPN per cc				
5.00	.00	.00	.01	.01	.02
2.50	.08	.16	.27	.47	.71
1.25	.23	.49	.77	.93	1.14
0	.34	.65	.89	1.19	1.28

B. *Effect of pH on the Inhibition of the Tryptic Digestion of Casein.* In order to study the effect of hydrogen ion concentration on the antitryptic behavior of heparin, 1 mg/cc of heparin was added to a 3% casein solution (buffered with N/10 phosphate) and the pH was adjusted to give the values shown in Table II, the final volumes being made the same in each case. The pH measurements were made with a glass electrode and a Beckman pH meter. One cc (= 0.5 mg) of a solution of trypsin was then added to 5 cc portions of the heparin-casein mixtures at 38°, and the NPN was determined after various time intervals as described in the preceding section. The results are shown in Table II. The optimum pH for digestion under these particular conditions appears to be shifted to the acid side of

TABLE II.
Effect of pH on Heparin-Inhibition of Tryptic Digestion of Casein.

pH	Time of incubation in min. 0′	10′	47′
	mg NPN per cc		
11.40	.33	.32	.32
9.40	.31	.60	1.10
7.90	.32	.70	1.15
7.00	.31	.69	1.20
5.90	.32	.56	.94

---

‡ Plaut Research Laboratories, Bloomfield, N. J. The preparation contained about 14% protein, the rest being $MgSO_4$.

the optimum for the tryptic digestion of casein in the absence of heparin (approximately pH 8.4).

C. *Rate of Thrombin Inactivation by Trypsin and Serum in the Presence of Heparin.* In a recent publication[3] we have shown that the rate of inactivation of thrombin (thrombinolysis) by trypsin is directly dependent on the concentration of trypsin, and may be characterized by a first order rate constant. This value may be determined quite readily by using the coagulation time of fibrinogen as a means of thrombin assay. The following equation may be used, provided the coagulation time is inversely proportional to the concentration of thrombin over the range studied:[3]

$$k = \frac{2.3}{(t_2 - t_1)} \log_{10} \frac{y_2}{y_1},$$

where k is the reaction rate constant and y_1 and y_2 are the observed coagulation times obtained with samples taken at times t_1 and t_2. The values for k in Table III represent the fraction of thrombin inactivated in one minute at 38°.

Table III A shows the effect of heparin on the rate constant which is obtained when a given amount of trypsin is added to thrombin. Trypsin alone will not coagulate purified fibrinogen,[4] and therefore does not interfere with the clotting test. The thrombin used was prepared by activating the isoelectric precipitate obtained on acidifying diluted plasma to pH 5.3, and then isolating the thrombin by acetone precipitation.[5] The negative k value in one case is probably due to the activation of a trace of prothrombin by the trypsin.[4]

TABLE III.
Effect of Heparin on Thrombinolysis.

	Heparin (μg/cc)	$k \times 10^4$ per min
A. 0.30 mg trypsin added to 5 cc of thrombin. pH = 6.9	1300	—15
	650	7
	320	210
	160	250
	0	437
B. An "unstable" thrombin preparation pH = 7.6	1670	76
	835	89
	417	107
	0	220
C. 0.8 cc of serum added to 5 cc of thrombin. pH = 7.4	1.20	224
	0.60	320
	0.30	326
	0.15	315
	0	407

---

[4] Eagle, H., and Harris, T. N., *J. Gen. Physiol.*, 1937, **20**, 543.

[5] Astrup, T., and Darling, S., *J. Biol. Chem.*, 1940, **133**, 761.

Table III B shows the results obtained with an "unstable" thrombin to which heparin was added, and Table III C shows the effect of heparin in retarding the "progressive" destruction of thrombin by fresh serum.[3] Ample evidence has already been presented for the presence of a tryptase in serum.[3, 6, 7, 8]

These results are wholly consistent with the data in section A. Heparin apparently decreases the rate of thrombinolysis because it interferes with the thrombin-tryptase interaction. This inhibiting action must not be confused with the *"immediate" antithrombic action* of heparin (in the presence of the accessory plasma factor) in preventing the thrombin-fibrinogen coagulation reaction.[9] However, it may be quite significant in the *antiprothrombic* action of heparin, since we know that trypsin and tissue extracts (which presumably contain tryptases) are capable of overcoming the inhibitory action of heparin in the first phase reactions.[10]

Summary. Heparin inhibits the action of trypsin and serum tryptase. The significance of this phenomenon in the blood clotting mechanism is briefly discussed.

11577

Detoxication of Benzoic Acid by Glucuronic Acid in Humans. Rate of Detoxication.

HARRY WAGREICH, ADOLPH ABRAMS AND BENJAMIN HARROW.
From the Department of Chemistry, The City College, College of the City of New York.

I. *Amount of Detoxication by Glucuronic Acid.* While there is definite evidence that in the pig, the dog and the sheep, the feeding of benzoic acid results in the elimination of hippuric acid and of benzoyl glucuronide,[1] showing detoxication with glycine and with glucuronic acid, in humans the results are conflicting. Brakefield,[2] for example, finds detoxication with glycine, but no detoxication

---

[6] Collingwood, B. J., and McMahon, M. T., *J. Physiol.*, 1913-14, **47**, 44.

[7] Schmitz, A., *Zeits. physiol. Chem.*, 1936, **244**, 89; 1937, **250**, 37.

[8] Jobling, J. W., and Peterson, W., *J. Exp. Med.*, 1914, **19**, 480.

[9] Glazko, A. J., and Greenberg, D. M., *Am. J. Physiol.*, 1940, **128**, 399.

[10] Ferguson, J. H., PROC. SOC. EXP. BIOL. AND MED., 1939, **42**, 33.

[1] Csonka, F. A., *J. Biol. Chem.*, 1924, **60**, 545; Brakefield, J. L., and Schmidt, C. L. A., *ibid.*, 1926, **67**, 523.

[2] Brakefield, J. L., *J. Biol. Chem.*, 1927, **74**, 783.

whatever with glucuronic acid. Quick,[3] on the other hand, claims a detoxication with glucuronic acid, which may, under certain conditions, amount to as high as 10 to 12%.

In the experiments of Brakefield and Quick only 2 subjects were used. Furthermore, the method of estimating the glucuronide, by applying reduction procedures, is open to question.[4]

Our work is an attempt to clear up this problem. In this investigation, as in a previous one,[5] we used the method of Maughan, Evelyn and Browne[6] to determine urinary glucuronides and obtained results in normal control urines consistent with those of other workers. In

TABLE I.
Amounts of Glucuronic Acid Eliminated.

Subject	Amt ingested, g	Mg glucuronic acid per 24-hr sample Days							Avg mg glucuronic acid per 24 hr in normal urine†	Increase in mg glucuronic acid on ingestion of indicated dose‡
		1	2	3	4*	5	6	7¶		
A	5	482	484	469	733	604	690	613	478	255
B	5	378	558	456	1022	468	426	436	464	558
C	5	461	582	527	826	622	537	493	523	303
D	5	432	572	483	972	729	646	568	496	476
E	5		444	559	688	590	532	547	501	187
F	5	522	494	576	1241	595	500	449	531	710
G	5	357	417	485	736	304	647	455	420	316
H	5	535	300	509	636	312	407	574	448	188
I	5	546	859	488	1272	773	795	762	631	641
J	5	410	372	478	861	525	522		420	441
K	5	569	559	520	899	501	562	554	549	350
L	6§	366	352	405	896	445	422	358	374	522
M	6	762	797	765	1141	773	795	762	775	366
M	7	Avg Norm. =		775	1205				775	430
M	8	Avg Norm. =		775	1750				775	975

*Ingestion of benzoic acid at the beginning of the fourth day.

†These figures were obtained by taking the average of the first 3 days.

‡These figures were obtained by subtracting the average of the first 3 days from the figure of the fourth day which represents the day of ingestion.

§It was not found easy to ingest more than 5 g of benzoic acid without developing nausea, headache, and a tendency to vomit. This explains the fewer subjects. Three subjects ingested 8 g of benzoic acid; 2 vomited within 25 minutes. The third was able to retain the benzoic acid; the results of the analyses on his urine samples are given in the table.

¶The amounts of glucuronic acid recorded in the table for the fifth, sixth, and seventh days are given as a comparison with the first 3 days to show that one day following the ingestion of benzoic acid the urinary glucuronides tend to return to within the subject's normal range.

---

[3] Quick, A. J., *J. Biol. Chem.*, 1931, **92**, 65.

[4] Harrow, B., and Sherwin, C. P., *A Text-Book of Biochemistry*, 1935, p. 380.

[5] Wagreich, H., Kamin, H., and Harrow, B., PROC. SOC. EXP. BIOL. AND MED., 1940, **43**, 468.

[6] Maughan, G. B., Evelyn, K. A., and Browne, J. S. L., *J. Biol. Chem.*, 1938, **126**, 567.

addition, we have also studied the rate of excretion of benzoyl glucuronide.

Experimental Procedure. Twenty-four-hour urine samples of normal male subjects on a normal diet were collected for 3 days. On the fourth day, the indicated amount of benzoic acid was ingested The benzoic acid was first neutralized with 0.35 M NaOH and diluted to 200 ml. The urine was collected during the following 4 days. The amount of glucuronide was determined in each 24-hour sample according to the method of Maughan, Evelyn, and Browne,[6] which makes use of the color developed with naphthoresorcinol and concentrated hydrochloric acid. The instrument used for analysis was a single-cell direct reading photoelectric colorimeter.

The results with 15 subjects are recorded in Table I.

Table II summarizes these results. It can be seen that an appreciable quantity of benzoic acid is excreted as the glucuronide in the human. The ingestion of from 5-7 g of benzoic acid gives rise to an excretion of about 5% in the form of its glucuronide. Greater per cent detoxication is apparently obtained with 8 g of benzoic acid. However, we do not want to stress this point inasmuch as we cite one example. We have already pointed out our difficulty and for that matter, the difficulty of other investigators, in getting subjects to ingest more than 5 g of benzoic acid.

II. *Rate of excretion of the glucuronide.* Urines were collected in 3-hour intervals before and after ingestion of the benzoic acid. Two individuals were given 5 g; two, 6 g; one, 7 g; and one, 8 g.

Fig. 1 shows 3 typical curves. It can be seen that the maximum amount of detoxication by glucuronic acid occurs in the first 3 hours after ingestion; and that with 5 and 6 g of benzoic acid, practically complete detoxication occurs within 9 hours. With 8 g of benzoic acid, complete detoxication does not occur before about 15 hours. It is interesting to note that Quick,[3] using one subject who ingested 8 g of benzoic acid, and making determinations at one-hour intervals, obtained maximum excretion of glucuronides in 2 hours.

Summary. (1) Contrary to Brakefield, and in agreement with

TABLE II.
Increase in Glucuronic Acid as a Result of Feeding Benzoic Acid.

Amt of benzoic acid ingested g	No. of cases	Avg increase in glucuronic acid in mg	% detoxication
5	11	402	5.0
6	2	444	4.7
7	1	430	3.9
8	1	975	7.7

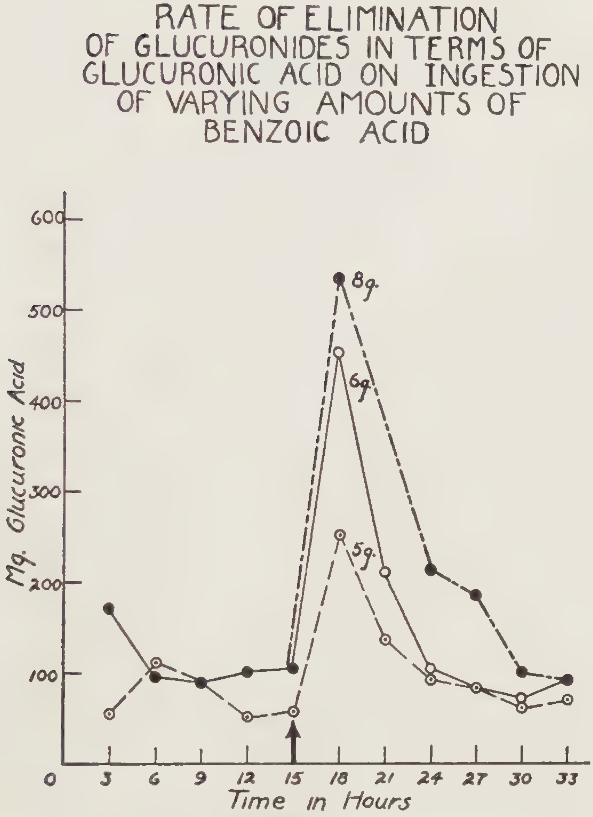


FIG. 1.

In each case, 5 three-hour normal samples of urine were taken. On the fifteenth hour, marked by an arrow on the graph, the benzoic acid was ingested.

The normals for the curves representing the 6 and 8 grams are the same because the same subject was used.

Quick, we find that in humans an appreciable quantity of benzoic acid is excreted as the glucuronide. The ingestion of 5, 6, and 7 g of benzoic acid leads to an excretion of some 5% in the form of its glucuronide. The ingestion of 8 g gives a higher figure (7.7%). (2) The maximum excretion occurs in the first 3 hours. Detoxication by glucuronic acid is complete in 9 to 15 hours, depending on the amount of benzoic acid ingested.

11578 P

Manganese as a Possible Factor Influencing the Occurrence of Lameness in Pigs.*

R. C. Miller, T. B. Keith, M. A. McCarty and W. T. S. Thorp.

From the Pennsylvania State College.

Impairment of the normal movement of the legs frequently occurs in swine. This condition is variously termed lameness, stiffness, posterior paralysis or incoördination, depending on its severity and etiology. A characteristic stiffness occurs in rickets in pigs, a condition in which there is abnormal bone development. Incoördination occurs in pigs as the result of vitamin A deficiency, in which there are degenerative changes in the central nervous system. In addition, however, lameness of one type or another occurs in some instances in which calcium, phosphorus, and vitamins A and D do not appear to be the limiting factors.

In the course of growth studies of pigs conducted by the authors during the past few years, a type of stiffness or lameness has been encountered which appears to be etiologically different from any of the forms described in the literature. This condition occurred in pigs which were fed similar rations, all of which were comprised of yellow corn, tankage, soy bean oil meal, ground alfalfa hay and salt. The pigs were fed individually and indoors but had access to direct sunlight at all times in outdoor paved pens.

The growth of the pigs was good but lameness occurred at about the time they reached a body weight of 150 pounds. The condition was at first characterized by a slightly halting gait, but it slowly progressed in severity until it was only with difficulty that the pigs could rise to their feet. The behavior of the pigs indicated that the condition was painful. Gross manifestations, in addition to stiffness, were enlarged hock joints and crooked legs which occurred even with some pigs which never became stiff. It should be noted that only 30 out of a total of 60 pigs receiving the above treatment were affected with stiffness. During the period of experiment 9 of the unaffected pigs did not attain the weight of 150 pounds, the weight at which stiffness usually occurred.

In outward appearance the condition resembled rickets. However, it was not alleviated or affected in any way by the addition of calcium and phosphorus to the ration or by the administration of cod liver oil and irradiated yeast. Subsequent analysis of the ulni and radii of 18 affected pigs showed that the mineral content of these bones was normal, the average value for bone ash being 58.92% on the dry, fat-free basis. Normal values were also obtained for the inorganic phosphorus content of the blood. However, there were certain constantly occurring changes observed in the leg bones which indicate abnormal development. Upon gross examination, the distal extremity of the radius and of the ulna showed enlargement. Roentgenographs of this area of these bones indicate that there is an increased activity of the tissues at the osteochondral junction, this area being definitely enlarged, but there was no apparent irregularity of the zone of ossification and no other changes present other than hypercalcification, thus eliminating the possibility of rickets.

---

* Authorized for publication on June 8, 1940, as paper No. 974 in the Journal Series of the Pennsylvania Agricultural Experiment Station.

The occurrence of stiffness was first noticed during the feeding of rations which had relatively high mineral contents. These ranged from 6 to 9% and suggest a point of similarity between these rations and those producing perosis in chicks. In view of the reported efficacy of manganese in preventing perosis, the swine rations were analyzed and found to have low manganese contents ranging from .0011 to .0014%.

Manganese sulfate was then added to the swine rations in amounts to supply 50-60 parts per million of manganese. As a result 15 pigs which received the manganese supplemented rations showed no signs of stiffness. Fifty percent of litter and pen mates of these same pigs fed the unsupplemented rations became stiff as usual. However, the addition of manganese to the ration was ineffective in curing stiffness after it had developed.

Hogan[1] has recently presented evidence to show that factors other than manganese may be involved in the prevention of perosis in chicks. The authors do not claim any similarity between perosis in chicks and the type of stiffness in swine described above, but wish to suggest a possible rôle of manganese in the nutrition of swine which apparently involves bone development.

---

[1] Hogan, A. G., Richardson, L. R., and Homer, Patrick, paper presented at the New Orleans Meeting of the American Institute of Nutrition, March 13, 1940.

11579 P

Influence of Ascorbic Acid on Oxidation of Tyrosine by Ultraviolet Light.

STEPHEN ROTHMAN. (Introduced by G. F. Dick.)

From the Department of Medicine, Section of Dermatology, and the Department of Pharmacology, University of Chicago.

In a previous communication[1] experiments were reported which indicated that there is a close similarity in melanin formation from tyrosine by tyrosinase and by ultraviolet light. The same similarity can be observed concerning the influence of ascorbic acid on this process.

The influence of ascorbic acid on oxidation and oxidation products of tyrosine is threefold. 1. Ascorbic acid markedly furthers dopa formation. It has been shown[2, 3] that in the tyrosine-tyrosinase system more dopa is formed if ascorbic acid is present. 2. Ascorbic acid prevents the further oxidation of dopa. The inhibitory effect of ascorbic acid on oxidation of poly-hydroxy-phenols by any kind of oxidative agent was observed as early as 1930.[4] 3. Melanin is transformed by ascorbic acid into a lighter product called "reduced melanin".[5, 6]

This third effect on melanin is due to the reducing power of ascorbic acid and can be performed with any reducing agent. However, the paradoxical combination of enhancement of tyrosine transformation to dopa and inhibition of further oxidation is a unique effect of ascorbic acid.

When studying the effects of ascorbic acid on oxidation of tyrosine by ultraviolet light the first effect observed was a very marked acceleration and increase of dopa formation. In one experiment the values were 7.2 mg % dopa with ascorbic acid and 2.9 mg % dopa without ascorbic acid after irradiation for 90 minutes. This effect was not simply due to the inhibition of melanin formation with a subsequent accumulation of dopa but it was proven to be a true catalysis of tyrosine oxidation as shown in the correspondingly faster decrease of tyrosine concentration.

After stopping the radiation and keeping the irradiated tyrosine

---

[1] Rothman, S., PROC. SOC. EXP. BIOL. AND MED., 1940, **44**, 485.

[2] Schaaf, F., *Helvet. Chim. Acta*, 1935, **18**, 1017.

[3] Evans, W. C., and Raper, H. S., *Biochem. J.*, 1937, **31**, 2155.

[4] Szent-Györgyi, A., *Science*, 1930, **72**, 125.

[5] Figge, F. H. J., PROC. SOC. EXP. BIOL. AND MED., 1939, **43**, 127; *J. Cell. and Comp. Physiol.*, 1940, **15**, 232.

[6] Miescher, G., and Minder, H., *Strahlentherapie*, 1939, **66**, 6.

TABLE I.

	Tyrosine irradiated 75 minutes with acetic acid	 ascorbic acid
Initial pH	2.8	2.8
Tyrosine mg %	47.8	43.8
Dopa mg %	3.1	7.0

ascorbic acid mixtures in the dark a rapid further increase of dopa concentration occurred. Concerning this effect it must be remembered that the transformation of tyrosine to dopa by ascorbic acid occurs also in the dark without addition of any oxidative agent.[2, 7] However, the dopa formation in the dark after irradiation was considerably faster than in non-irradiated samples. Apparently ascorbic acid acts in this process as a transmitter of atmospheric oxygen and the reaction is markedly accelerated by ultraviolet light. In a relatively short time one-half the amount of tyrosine in a 50 mg % solution could be transformed into dopa by light and ascorbic acid.

Not only tyrosine but any para-mono-hydroxy-phenyl derivative is transformed into the corresponding catechol derivative by ascorbic acid. Thus synephrin is transformed into epinephrine. Highest epinephrine concentrations can be obtained by irradiation of synephrin with ascorbic acid but considerable amounts are also formed if the synephrin-ascorbic acid mixture is kept in the dark for 2-3 weeks. Such a transformation is known to occur also by ultraviolet irradiation alone.[8, 9] However, in this case, the epinephrine formed by ultraviolet light undergoes further oxidation and destruction, so that with light alone it is not possible to produce those high concentrations which are formed in presence of ascorbic acid stabilizing the catechol derivative and preventing further oxidation.

The stabilization of dopa by ascorbic acid and the prevention of further oxidation by irradiation are absolute if an excess of ascorbic acid is constantly present during the exposure to ultraviolet light. These findings indicate that any kind of physiologic or pathologic pigmentation can be prevented by ascorbic acid if its concentration in the tissue is sufficiently high.

The presence of ascorbic acid in the suprarenal medulla may have a double physiologic importance: (1) formation of catechol precursors of epinephrine from phenol compounds of the proteins (phenylalanin, tyrosine) and (2) stabilization of the epinephrine after it is formed in the suprarenal glands.

The effect of ascorbic acid and other reducing agents on melanin

---

[7] Abderhalden, E., *Fermentforschung,* 1936, **15**, 24.

[8] Ewing, P. L., *J. Lab. and Clin. Science,* 1934, **20**, 16.

[9] Konzett, H., and Weis, W., *Arch. f. exp. Path. und Pharm.,* 1939, **193**, 440.

formed by actinic oxidation of tyrosine is characterized (1) by rapid lightening of the color and (2) by formation of a yellow water-soluble substance from the colloidal particles of melanin. The "melanolysis" proceeds at a more rapid rate as the pH is lowered from 7.4 to 2.0. However, it also occurs at physiological pH's. This change might be of importance in the depigmentation of patients with Addison's disease treated with ascorbic acid.[4] The same effect has been obtained in pieces of skin from Negroes.

Summary. Ascorbic acid furthers the actinic transformation of tyrosine into dopa and that of synephrin into epinephrine but inhibits any further oxidation. It reduces melanin to a water-soluble yellow substance.

11580

Excretion of Androgens and Estrogens in Males with Mammary Carcinoma.*

NAOMI YOLTON AND CHARLES REA. (Introduced by M. B. Visscher.)

From the Departments of Physiology and Surgery, University of Minnesota.

We have investigated the excretion of androgens and estrogens in 2 cases of male mammary carcinoma. The results obtained are recorded in Table I.

TABLE I.
Excretion of Androgens and Estrogens in Patients with Mammary Carcinoma.

Case	Sex	Age	Diagnosis	Androgens in mg of Androsterone per 24 hr	Estrogens in γ of Estrone per 24 hr
P.S. No. 658182	M	80	Scirrhous Carcinoma (Breast)	3.4 8.1 3.7 9.4 5.6 9.9 5.4	0.8-1.04 for 6 samples
A.A. No. 667313	M	78	Adeno-Carcinoma (Breast)	5.1 4.1 8.8	<1.25 <2.5
F.G. No. 694278	M	75	Control for Age and Sex (Ileostomy)	6.4 4.7 7.3 9.2 10.1	
S.W. No. 675993	M	66	Control for Age and Sex (Skin Graft)	6.7 10.0 11.4 7.2	
H.D. No. 683167	F	45	Scirrhous Carcinoma (Breast)	4.4 4.1 3.8 5.0 4.5 6.2	2.5-5.0 <5.0
E.K. No. 642071	F	52	Scirrhous Carcinoma (Breast)	11.8 10.3 10.0	
O.H. No. 686366	F	53	Control for Age and Sex (Chronic Cholecystitis)	5.2 6.2 5.4 5.2	

---

* Aided by a grant from the Cancer Institute of the Medical School of the University of Minnesota.

Twenty-four-hour urine specimens were extracted and fractionated according to the method described by Gallagher, Peterson, Dorfman, Kenyon, and Koch.[1] Samples were assayed for their estrogenic potency by the vaginal smear technic, using mice. Androgenic† assays were done by a modification of the Zimmerman method as described by Friedgood and Whidden.[2] We endeavored to correct for the inherent color of the samples by subtracting the equivalent of the reading obtained when an aliquot (equal in amount to that used for the actual determination) was treated with all the reagents except m-dinitrobenzene. This method gives values higher than those which would be obtained by biologic assay, but we feel that it is probably acceptable for use in a comparative study.

It will be seen that the male patients with mammary carcinoma did not differ significantly from the controls in regard to the amount of androgens and estrogens excreted. The low excretion of both androgens and estrogens in the aged, found by several previous investigators, and the daily variability in the excretion of androgens reported by Gallagher, *et al.*,[1] are confirmed.

We are indebted to Drs. M. B. Visscher, L. T. Samuels, and L. Earle Arnow for their suggestions and coöperation.

11581

Comparative Activities of Certain Antihemorrhagic Compounds.

H. J. ALMQUIST AND A. A. KLOSE.

From the Division of Poultry Husbandry, University of California, College of Agriculture, Berkeley.

Various quinones with vitamin K activity have been assayed in order to establish the comparative potencies of these compounds in relation to a common reference standard. The reference standard is an hexane extract of dried alfalfa equivalent in potency to 1 g of dried alfalfa per cc. This reference standard at 2 or more levels has been employed in all of our assays for the past 2 years. A secondary reference standard consisting of a sample of dried alfalfa has also been preserved. Neither of these standards has shown any loss of total or relative activity. In periodic assays the potency of the second reference standard has been obtained as 1.09, 1.03, 1.07, and 1.06 cc of the first standard per gram.

---

[1] Gallagher, T. F., Peterson, D. H., Dorfman, R. I., Kenyon, A. T., and Koch, F. C., *J. Clin. Invest.*, 1937, **16**, 695.

† Grateful acknowledgment is made to Ciba Pharmaceutical Products and to Schering Corporation for the crystalline androsterone; and to Parke-Davis for crystalline estrone.

[2] Friedgood, H. B., and Whidden, H. L., *Endocrin.*, 1939, **25**, 919.

Some assays have been conducted as we have already described,[1] that is, by placing the supplement in the diet of depleted chicks and determining the prothrombin clotting time after 7 or more days. More recently,[2] after a depletion period of 10 to 14 days, we have given the supplement orally for 4 days and have determined prothrombin clotting time on the fifth day. Certain supplements have been assayed by both procedures and have given identical results. In a few cases, where evidence of loss of the supplement in the diet was encountered, the oral feeding was relied upon entirely. In all asays, the reference standard was employed at 2 or more levels in order to establish values for interpolation and calculation of potencies in terms of the standard. This may be done by means of the linear relation between the reciprocal of the prothrombin clotting time and the logarithm of the vitamin K dosage.[1] No "master curves," "response curves" or arbitrarily fixed values are required in our method. The results of the assays, which are in most cases average values of several determinations, are given in Table I . As a further illustration of the assay procedure, results of one recent assay are given in detail in Table II.

The activities listed in Table II agree substantially with those in Table I for the same compounds. The value for the sample of 2-methyl-4-amino-1-naphthol hydrochloride, corrected for the ethanol of crystallization, becomes 97,000, the same as that in Table I for this compound without ethanol of crystallization.

The activity of 2-methyl-1,4-naphthoquinone is the same whether administered orally in water or ethyl laurate solution (Table II). This has been our repeated experience. However, Dann[3] reported that 2-methyl-1,4-naphthoquinone is 3 times as potent when administered orally in oil as compared to water, while Ansbacher, Fernholz and Dolliver[4] state that it is twice as potent in water as in oil.

The tetra-sodium salt of 2-methyl-1,4-naphthohydroquinone di-

---

[1] Almquist, H. J., and Klose, A. A., *Biochem. J.*, 1939, **33**, 1055.

[2] Almquist, H. J., and Klose, A. A., *J. Biol. Chem.*, 1939, **130**, 787.

[3] Dann, F. P., PROC. SOC. EXP. BIOL. AND MED., 1939, **42**, 663.

[4] Ansbacher, S., Fernholz, E., and Dolliver, M. A., PROC. SOC. EXP. BIOL. AND MED., 1940, **43**, 652.

TABLE I.
Antihemorrhagic Activities of Certain Derivatives of 2-methyl-1,4-naphthoquinone.

Compound	Reference standard per g, cc	Source
2-methyl-1,4-naphthoquinone	205,000	Authors
2-methyl-1,4-naphthoquinone, dimerized	14,000	F. Giral
2-methyl-1,4-naphthohydroquinone	190,000	Authors
2-methyl-1,4-naphthohydroquinone, diacetate	92,000	"
2-methyl-3-hydroxy-1,4-naphthoquinone (Phthiocol)	500	R. J. Anderson
2-methyl-3-acetoxy-1,4-naphthoquinone	900	Authors
2-methyl-3-amino-1,4-naphthoquinone	3,000	"
2-methyl-3-nitro-1,4-naphthoquinone	800	"
2-methyl-3-palmityl-1,4-naphthoquinone	25,000	"
2,3-dimethyl-1,4-naphthoquinone	5,400	Merck & Co.
2-methyl-4-amino-1-naphthol hydrochloride	97,000	E. A. Doisy
2-methyl-1,4-naphthohydroquinone diphosphoric acid ester (tetra sodium salt + 6 molecules water)	101,000	Hoffman-LaRoche
2-methyl-3-phytyl-1,4 naphthoquinone (vitamin K_1)		
natural	62,700	P. Karrer
"	61,600	E. A. Doisy
"	55,000	Authors
" , diacetate	20,000	E. A. Doisy
synthetic	57,000	"
"	56,000	Authors
"	59,000	Merck & Co.
2-methyl-3-(?)-1,4-naphthoquinone (vitamin K_2)	50,000	E. A. Doisy

TABLE II.
Comparative Assay of Antihemorrhagic Compounds by 4-day Oral Administration to Depleted Chicks.

Supplement In ethyl laurate solution	Daily dose	No. chicks	Avg prothrombin clotting time, sec	Reference standard per g, cc
Reference standard	0.15 cc	9	31.7	—
2-methyl-1,4-naphthoquinone	0.5 γ	10	37.5	191,000
" "	2.0 γ	10	22.7	
In water solution				
2-methyl-1,4-naphthoquinone	0.5 γ	10	39.9	187,000
" "	2.0 γ	10	22.9	
2-methyl-4-amino-1-naphthol hydrochloride (½ molecule ethanol)	3.0 γ	9	25.8	87,500
2-methyl-1,4-naphthohydroquinone diphosphoric acid ester (tetra sodium salt + 6 molecules water)	3.0 γ	10	24.9	95,700

phosphoric acid ester is of particular interest because our results show, in agreement with the report of Foster, Lee and Solmssen,[5] that on a molecular basis this compound is more potent than the methyl naphthoquinone. Potencies for the compound as administered (containing 6 molecules of water of crystallization) were found to be 101,000 and 95,700 in two assays. On the molecular basis, these values would be 310,000 and 294,000 respectively or 1.48 and 1.54 times the activity of the methyl naphthoquinone used in the same assay. There remains no doubt that this is the most potent antihemorrhagic compound yet prepared. The suggestion[5] that this phosphoric acid ester may represent a step in the metabolism of the methyl naphthoquinone seems to be logical. On the other hand, Ansbacher, *et al.*,[4] have claimed that this di-phosphoric acid ester is only 1/20 as potent as the methyl naphthoquinone.

---

[5] Foster, R. H. K., Lee, J., and Solmssen, U. V., *J. Am. Chem. Soc.*, 1940, **62**, 453.

The 2-methyl-1,4-naphthohydroquinone diacetate (92,000) calculated to the 2-methyl-1,4-naphthoquinone basis has a corrected potency (124,000) which is much less than that of the methyl naphthoquinone. Similarly the diacetate of dihydro-vitamin K_1 (20,000) has a corrected potency of 22,600 which is a little more than one-third the potency of the original preparation of vitamin K_1 (61,600). Our results agree qualitatively with those of Binkley, *et al.*,[6] in respect to this potency-lowering effect of reductive acetylation.

The activity of the water-soluble phenol, 2-methyl-1,4-naphthohydroquinone (190,000), which is spontaneously oxidized by air to the methyl naphthoquinone is not appreciably different from that of the latter, probably because the phenol is converted to the naphthoquinone before or soon after absorption. In this respect our results agree with those of Thayer, *et al.*[7]

Dimerization of the methyl naphthoquinone as catalysed by light involves 2,3 condensation. The condensed product which we obtained from Dr. F. Giral exhibited a potency (14,000) corresponding to only a few percent of unchanged methyl naphthoquinone. It was known to be not quite pure. The pure dimer is probably completely inactive. This result is of interest in relation to the proposed use of 2-methyl-1,4-naphthoquinone as a standard and to its stability as such.

In two joint assays of preparations of vitamins K_1 and K_2 obtained from Professor E. A. Doisy we found the same potency ratio, 1.25, which is almost exactly the inverse molecular ratio of these compounds or the direct ratio of their respective contents of methyl naphthoquinone. This observation is most easily explained by assuming that in each case the long side chain of these compounds is split off to the same extent, perhaps completely.

---

[6] Binkley, S. B., Cheney, L. C., Holcomb, W. F., MacCorquodale, D. W., Thayer, S. A., and Doisy, E. A., 1939, 98th Meeting, Am. Chem. Soc., Boston.

[7] Thayer, S. A., Binkley, S. B., MacCorquodale, D. W., Doisy, E. A., Emmett, A. D., Brown, A. R., and Bird, O. D., *J. Am. Chem. Soc.*, 1939, **61**. 2563.

If this view of the metabolism of vitamins K_1 and K_2 is correct, the potencies of these compounds can not be greater than that of their corresponding content of the methyl naphthoquinone. Their potency is actually less since we obtain, after repeated assays, a potency for 1 milligram of vitamin K_1 equal to that of 300 μg of 2-methyl-1,4-naphthoquinone, whereas it actually contains 380 μg. Correspondingly, the potency of vitamin K_2 appears to be 240 μg out of a theoretical 307.

The phytyl side chain of vitamin K_1 and the larger side chain of vitamin K_2 seems nonspecific, as indicated by the fact that the methyl naphthoquinone itself is more active without these side chains. Furthermore, a side chain of an entirely different character, the palmityl group, also gives rise to a compound with fairly high activity (Table I).

The phthiocol used in our assays was in some instances submitted to a further purification by washing a solution of phthiocol in dilute sodium bicarbonate repeatedly with ethyl ether. The potency of the phthiocol was unchanged by this treatment. The ratio of its potency to that of the methyl naphthoquinone is 1 to 400 according to our assay values. Flynn and Warner[8] reported a potency ratio 1 to 500 for these compounds. Fernholz and Ansbacher[9] have claimed, however, that the potency ratio may be as low as 1 to 4000.

The antihemorrhagic activity of 1 cc of our reference standard is equivalent to that of 5 μg of 2-methyl-1,4-naphthoquinone. All of our values are convertible on this basis since the reference standard has been used in all assays pending the time when a universal standard might be adopted.

---

[8] Flynn, J. E., and Warner, E. D., PROC. SOC. EXP. BIOL. AND MED., 1940, **43**, 190.

[9] Fernholz, E., and Ansbacher, S., *Science*, 1939, **90**, 315.

11582

Androgen in the Woodchuck Hibernating Gland.

J. E. Sweet* and W. H. Hoskins.†

From the Department of Surgery and the Barbara Henry Research Laboratory, Department of Medicine, New York Hospital, and Cornell University Medical College, New York.

During the summer of 1939 it was possible for one of us to collect the perfectly fresh tissue from a number of woodchucks *(Marmota monax)*.‡ The tissues obtained immediately after the animals' death were placed in suitable fixing solution for histological study, while the so-called hibernating glands were chopped with scissors into small bits and placed in absolute alcohol containing 1.5% hydrochloric acid. The hibernating gland present in many hibernating and a few non-hibernating mammals has attracted the attention of a number of investigators but has yielded remarkably little information as to its function. The distinct histological character of the glandular adipose tissue as compared with ordinary fat seems to be established.[1] In the woodchuck three-fourths of the body fat is lost during hibernation while the hibernating gland is not utilized to any extent during this period. During the 3 to 4 weeks following emergence from hibernation, which is said to be the mating season, the hibernating gland shrinks rapidly to one-fourth of its original weight, while all but a trace of the body fat is lost. The manner in which this tissue is spared during the hibernating period and utilized during the breeding season has not been explained.

During the course of these dissections an impression was gained that some material giving rise to the brownish color of the hibernating glands was being deposited in pre-existing fat lobules, rather than that the hibernating gland is a tissue entirely distinct from the neighboring fat.

In one old male animal the testes presented a striking, almost black, color. There appeared to be some of this same pigmented material around the urethra and also in the adrenals.

The question arose in our minds as to whether some pigmented substance was being elaborated by the interstitial cells of the testis and being stored in the hibernating glands; it is known that in the true hibernating animals the interstitial cells are apparently very active during the summer, while the spermatogenetic tissue seems to have almost disappeared. This idea occurred to us after the tissue had been brought back to the laboratory, and we realized that we did not have adequate controls for anything other than a preliminary experiment. However, we felt justified in trying a bioassay of the material for androgenic content, and the result is so striking that we feel free to present a preliminary communication, again emphasizing our appreciation of the incomplete character of the study.

---

* Professor of Surgical Research.

† Barbara Henry Research Fellow.

‡ We are greatly indebted to Dr. and Mrs. George H. Kittell, of Jamaica, L. I., expert riflemen, for this opportunity.

[1] Rasmussen, A. T., *J. Morph.*, 1923, **38**, 147.

The available tissue represented some 12 animals, both males and females. Unfortunately the glands were not kept separate as to sex. Eight months after collection the alcohol was poured off, the glands weighed (53 g), hashed, and extracted repeatedly with 95% alcohol on the steam bath. The tissue residue was then extracted for two 6-hour periods with ether in a Soxhlet extractor. The combined alcohol-ether extracts, including the original acid-alcohol solution, were evaporated and the residue dissolved in acetone, the solution chilled to remove phospholipins and the acetone evaporated. The residue was distributed between 70% alcohol and petroleum ether and both fractions evaporated. The weight of the petroleum ether fraction was 22.0 g, the 70% alcohol fraction 2.5 g. Both were assayed for androgen on the capon comb by the inunction method in parallel with a solution of pure androsterone as a standard. The petroleum ether fraction was inert. The alcohol fraction contained the equivalent of 110 μg (0.110 mg) androsterone in the 53 g of tissue. This is equal to 1 I.U. (International Unit) of androgen (100 μg of androsterone) in 50 g of tissue. Bull testis, the richest tissue source of androgen yet reported, contains 1 I.U. in 50-150 g.[2] Due to dehydration by the alcohol the fresh weight of the hibernating glands was doubtless appreciably greater than the 53 g noted but the amount of androgen found places this tissue within the range of the findings in bull testis.

It is impossible to evaluate this finding until other tissues of the woodchuck can be assayed for androgen. Its presence or absence in the body fat would be of special interest. Since we were unable to obtain more woodchuck material we investigated the androgen content of human male and female axillary fat.§ Although small amounts of androgen were found, equivalent to 1 I.U. in 1 kg of male (age 46) and 1 I.U. in 3 kg of female (age 55) axillary fat, these concentrations are so low as to be of questionable significance. (The age of the subjects conceivably may have been responsible for the low androgen content.) This marked difference in androgen concentration between the woodchuck hibernating gland and human axillary fat suggests that a high androgen content is not a property of all fatty tissues but may reflect the part played by the hibernating gland in the sex cycle of the woodchuck.

---

[2] Koch, F. C., *Physiol. Rev.*, 1937, **17**, 153.

§ We are grateful to Dr. Jacob Furth, Department of Pathology, The New York Hospital, and to Dr. Jacob Werne, St. John's Hospital, Queens, N. Y., Assistant Medical Examiner of the Borough of Queens, for securing this material.

Any theorizing as to the significance of the androgen in the woodchuck glandular adipose tissue must await confirmation of this finding and a study of its concentration in other tissues of the animal. In view of the functions already attributed to the hibernating gland: a protein-sparer;[3] a storage point for vitamins and lipoids;[4] and the source of a metabolism-depressing secretion,[5] we hesitate to attach any functional significance to the presence of androgen in the gland until further evidence is available.

Summary. The hibernating glands of woodchucks killed during the summer contained 1 International Unit of androgen, (100 μg androsterone) in 50 g of tissue, equal to the concentration in bull testis, the richest tissue source known. Human male and female axillary fat contained 1 International Unit in 1 kg and 3 kg respectively.

11583

A Laboratory Method for the Soilless Growth of Grass.

CLAIRE FOSTER, JAMES H. JONES, DORIS RUSSELL AND FRIEDA DORFMAN. (Introduced by D. W. Wilson.)

From the Departments of Pediatrics and Physiological Chemistry, University of Pennsylvania, and Children's Hospital of Philadelphia.

Fresh green plant material is frequently used to supplement standard stock diets in the raising of laboratory animals such as the rat. Recently, additional impetus has been given to the study of young green growing plants by observations on the previously unknown factor (factor *pl* of Wulzen and Bahrs,[1] grass juice factor of

---

[3] Vignes, H., *C. R. Soc. Biol.*, 1913, **75**, II, 360, 397, 418.

[4] Cramer, W., *Brit. J. Exp. Path.*, 1920, **1**, 184.

[5] Wendt, C. F., *Z. physiol. Chem.*, 1937, **249**, 2.

[1] Wulzen, R., and Bahrs, A. M., *Physiol. Zool.*, 1936, **9**, 508.

Kohler, *et al.*[2, 3, 4]) necessary for the normal growth of guinea pigs[1-5] and on the nutritive value of cereal grasses.[6] Such studies would be greatly facilitated if it should be possible to grow young plants in the laboratory under reproducible conditions at any time and in the desired quantities.

A method has been developed in this laboratory for the purpose of adding the grass juice factor to relatively purified breeding diets for nutrition studies on mice which are handled with bacteriological precautions. The following qualities are essential: (a) a product which will provide a potent source of the grass juice factor, (b) uniform seed, (c) a nutrient solution composed of chemically pure ingredients, (d) a planting base which is as inert as possible, and (e) general cleanliness, with emphasis on freedom from insects, vermin and harmful bacteria.

After more than a year of study, the following method has been evolved and has proved satisfactory. It consists essentially of spreading soaked seeds on a layer of cotton placed on a screen which has been suspended over a pan containing nutrient solution. The roots grow down through the cotton. The grass begins to sprout within 24 hours after planting, and reaches a height of about 5 inches by the seventh day (Fig. 1).

Before outlining the steps in the procedure, some essential features of the materials, equipment and environment suited to growing grass in the laboratory should be considered.

Seed. English rye grass was used during the first year but was discarded because it proved to be an unreliable source of the grass juice factor when assayed by the method of Kohler, *et al.*[4] Barley seed was next tried and was found to be satisfactory. A sufficient supply of first quality, fumigated seed for a series of experiments to be compared is desirable. It should be kept in a vermin-proof, cool, dry place.

Nutrient Solution. Culture Solution No. 1 was selected from the 8 formulas suggested by Ellis and Swaney[7] because its ingredients

---

[2] Kohler, G. O., Elvehjem, C. A., and Hart, E. B., *J. Nutr.*, 1937, **14**, 131.

[3] Kohler, G. O., Elvehjem, C. A., and Hart, E. B., *J. Nutr.*, 1938, **15**, 445.

[4] Kohler, G. O., Randle, S. B., Elvehjem, C. A., and Hart, E. B., PROC. SOC. EXP. BIOL. AND MED., 1939, **40**, 154.

[5] Cannon, M. D., and Emerson, G. A., *J. Nutr.*, 1939, **18**, 155.

[6] Graham, W. R., Jr., Kohler, G. O., and Schnabel, C. F., paper read at Cincinnati Meeting of Am. Chem. Soc., Apr. 8 to 12, 1940.

[7] Ellis, C., and Swaney, M. W., *Soilless Growth of Plants*, Reinhold Publishing Company, 1938.

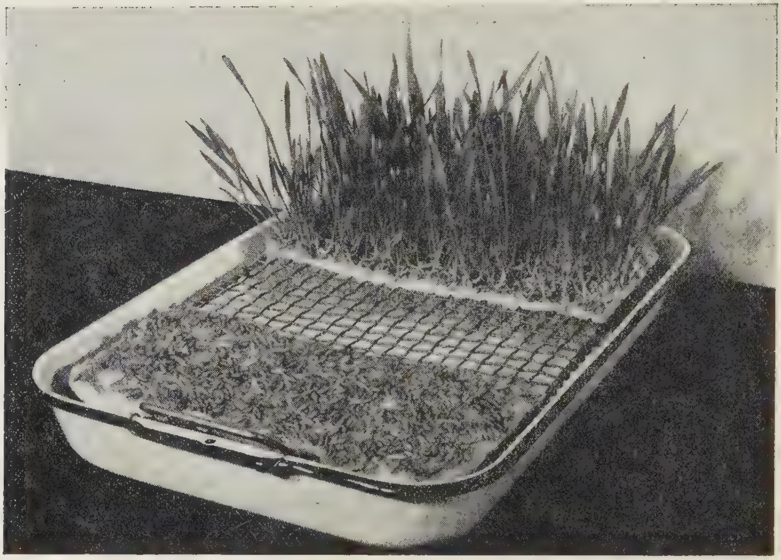


FIG. 1.

Planting pan with screen in place, showing seeds on day of planting and growing grass on the 7th day after planting.

are easily procured and it appeared to be equally adequate and less complicated than some of the others.

Planting Pans. Porcelain butcher trays 12″ wide, 17″ long and 2¼″ deep, of standard stock, are used. These trays are small enough to be easily handled, and large enough to grow about 75 g of barley grass per tray when it is cut on the seventh day.

Planting Screens. Cellar window wire which was galvanized, cadmium-plated or enameled with various finishes was tried and discarded because of corrosion. The corrosion not only contaminated the grass, but markedly retarded its growth. Stainless steel seems to be the material of choice at present. Two other qualities of the screen are important: (a) sufficient rigidity to prevent sagging, and (b) ample open space in relation to the wire to avoid interference with root growth. The stainless steel screens are half-inch mesh, and were made to fit the pans.*

Suspension Wires. 12-gauge aluminum wire is used for suspension of the screens because it is flexible enough to be adjustable and yet rigid enough to hold the screen in place. The need for this wire will be clear when the procedure for growing grass is considered below.

Cotton. Pieces from a pound roll of the cheapest absorbent cotton are cut to fit the planting screens. Since a layer of one-half the original thickness is sufficient, each piece of cotton is divided horizontally, so that it furnishes material for 2 screens.

---

* Made by H. T. Hernberg, 2012 North 32nd Street, Philadelphia.

Towel Supports. Galvanized iron cellar window wire cut in pieces 12¾″ x 18″ is used.

Towels. Dampened turkish towels, 24″ x 40″, have been found satisfactory for keeping the seeds moist on the first and second days after planting.

Temperature and Humidity. The optimum temperature and humidity have not been determined. It is evident, however, that a temperature below 70°F and a relative humidity of less than 40 percent definitely retard the development of grass grown in this manner.

Light. The light that comes through ordinary glass appears to be adequate. It is important that the grass be uniformly exposed; therefore, the pans are turned around daily.

Procedure (for barley seed and the equipment described above).

Day before planting: Place 70 g of barley seed and 140 cc of distilled water in a container, mix thoroughly and allow to stand at room temperature for 18 to 24 hours.

Day of planting: 1. Lay cotton on the stainless steel screen, pour distilled water over it, patting gently so that all the cotton will become quickly saturated. 2. Spread the seeds evenly over the cotton. 3. Lay the towel support over the pan, resting it on the edges. This is necessary to prevent the damp seeds from adhering to the towel. 4. Place the double-folded towel, which has been lightly wrung out in distilled water, over the pan, taking care to cover the pan completely in order to prevent drying out of the seeds. No amount of soaking after the first 48 hours will fully overcome the ill effects of drying the seeds during the first 2 days.

1st day after planting: 1. Uncover the pan and sprinkle the seed-covered cotton generously with distilled water. 2. Replace towel support and towel which is moistened as on the previous day.

2nd day after planting: 1. Uncover the pan and sprinkle as on the preceding day. 2. Pour water from the pan. 3. Using the aluminum suspension wires, suspend the screen about 2 inches above the bottom of the pan (adjust the wires over the extended rims on the ends of the pan and bend both ends of each wire into a hook to grip the screen). 4. Pour nutrient solution into the pan to within about 1 cm of the screen.

3rd to 6th days after planting: 1. Sprinkle as on the preceding day. 2. Keep nutrient solution at about the same level by adding it daily. 3. Keep screen raised so that roots clear the bottom of the pan.

7th day after planting: Cut and feed the green portion of the plants. In order to insure a uniform supply of fresh grass, only the first cutting is used. Seeds are planted at intervals to correspond to the days on which the grass is to be fed.

Kohler, Elvehjem and Hart[3] reported one experiment in which 5 g of greenhouse-grown oat grass per guinea pig per day did not furnish appreciable amounts of the grass juice factor. However, we have found that grass grown as described above and fed *ad libitum* in addition to the basal diet of Kohler and associates[4] completely protected guinea pigs against a deficiency of this dietary essential. Details of these experiments will be reported at a later time.

Although we were particularly interested in having a regular supply of the grass juice factor, grass for various purposes may be grown by this method.

11584

Effect of Rammstedt Operation on Incidence of Cinchophen Ulcer.

M. W. DAVIS, W. B. BRADLEY, W. H. BACHRACH AND A. C. IVY.
From the Department of Physiology and Pharmacology, Northwestern University Medical School, Chicago.

This study of the effect of the Rammstedt operation on the incidence of gastro-duodenal ulcer caused by the administration of large doses of cinchophen was undertaken for several reasons. First, it was observed that the ulcer caused by cinchophen is frequently located at the site of the pyloric sphincter, as well as in the duodenum, and along the lesser curvature of the stomach as has been observed by others.[1] Second, it was found that the intravenous injection of from 10 to 25 mg per kilo body weight of cinchophen caused an increase in the activity of the pyloric sphincter in 6 out of 14 dogs. The activity of the pylorus was recorded by a balloon made for the purpose. The increased activity of the pyloric sphincter, illustrated in Fig. 1, was abolished by atropine, but not by bilateral section of the vagi in the neck. Third, the results of studies by us on the effect of cinchophen (100 mg per kilo per day) on bilirubin clearance (4

---

[1] Churchill and Van Wagoner, *Arch. Path.*, 1932, **14**, 860; Bollman and Mann, *Proc. Staff Mayo Clin.*, 1935, **10**, 580.

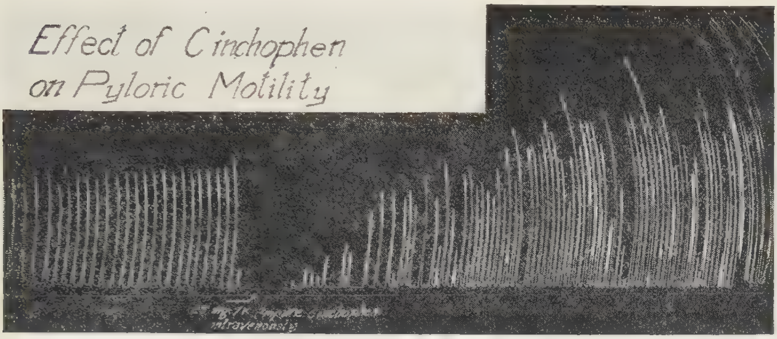


FIG. 1.

dogs) and serum phosphatase (4 dogs) failed to reveal any evidence of liver damage which might account for the ulcers. Fourth, when the excretion of cinchophen into gastric juice or pyloric secretion was examined by the method of Bradley,[2] a maximum excretion of 1.5 mg per hour was obtained. This was done to ascertain whether circulating cinchophen might be excreted into the lumen of the stomach in quantities sufficient to irritate, since it has been found that cinchophen introduced by routes other than the oral causes gastro-duodenal ulcer.[3, 4] Finally, we failed to observe a significant change in the gastric secretory response to a test meal in 3 Pavlov and 4 Heidenhain pouch dogs to occur after the daily administration of 100 mg of cinchophen per kilo body weight, though 5 of the animals developed ulcer. The averaged results are shown in Table I. These results do not necessarily disagree with those of Stalker, Bollman and Mann[5] who observed a hypercontinuous secretion rather than an increased response to a meal. We did not follow the output of secretion during 24 hours as they did.

The observations just referred to suggested that if a Rammstedt operation was performed, it might prevent ulcers from occurring at the site of the pyloric sphincter by preventing pylorospasm and might reduce the incidence of ulcer in the stomach by tending to promote enterogastric regurgitation and gastric evacuation.

Methods. The Rammstedt operation was performed by the usual surgical technic, the muscle being separated widely from the mucosa. Those dogs in which the mucosa was accidentally incised were discarded from the series.

---

[2] Bradley, W. B., to be published.

[3] Churchill and Manshardt, PROC. SOC. EXP. BIOL. AND MED., 1932, **30**, 825.

[4] Stalker, Bollman and Mann, *Arch. Surg.*, 1937, **35**, 290.

[5] Stalker, Bollman and Mann, *Arch. Surg.*, 1937, **34**, 1172.

TABLE I.
Effect of Cinchophen "B" on Gastric Secretory Response to a Meal in Pavlov and Heidenhain Pouch Dogs.

	Control Avg of 7-11 test meals		1st week		2nd week		3rd week		4th week		5th week	
Dog No.	Vol. cc	cc of N/10 HCl	Vol. cc	cc of N/10 HCl	Vol. cc	cc of HCl N/10	Vol. cc	cc of HCl N/10	Vol. cc	cc of HCl N/10	Vol. cc	cc of HCl N/10
1	42.8	37.0			39.8	43.1						
2	79.7	87.4			79.1	86.9						
3*	36.9	32.3	21.2	14.9	20.0	15.5						
4*	19.7	6.4	15.2	5.9	15.2	5.9	24.3	11.1				
5*	19.5	8.2	19.7	7.1	21.6	6.0	27.0	8.2	18.1	5.3		
6*	12.0	7.9	6.6	2.0	22.2	10.5	14.3	7.2				
7*	12.6	8.1	14.4	6.3	22.0	11.5	16.8	7.5	13.7	7.8	11.1	7.1

*These dogs had an ulcer at autopsy.

Dogs 1, 2 and 3 were typical Pavlov pouches and Dogs 4, 5, 6 and 7 were Hendenhain pouches, according to the curve of secretion. 2 to 5 test meals were given per week.

A maximum of 1.5 mg of cinchophen was secreted per hour in the gastric juice. For Dog No.:

1. No ulcer. 6 test meals. 1.5 mg cinchophen secreted in juice per hour.
2. ,, ,, 5 ,, ,, Max. of 1.5 mg cinchophen secreted in juice per hr.
3. 4 test meals each week. Ulcer at autopsy.
4. 5 ,, ,, ,, ,, ,, ,, ,,
5. 2 ,, ,, ,, ,, ,, ,, ,,
6. 2 ,, ,, ,, ,, ,, ,, ,,
7. 2 or 3 test meals each week. Ulcer at autopsy.

Two different cinchophen preparations were used. Cinchophen "A" had physical properties analogous to the cinchophen which was used in ulcer studies prior to 1935,[1, 3, 6] and was available on the market at that time. It had a greyish cream color and contained some phenolquinolin and an alkali-insoluble residue. Cinchophen "B" was a white powder and contained no impurities that we could detect. The cinchophen preparation used in our laboratory prior to 1935 was apparently more toxic than cinchophen "B", in that it produced lethal effects more rapidly. For example, 100 mg per kilo daily of the cinchophen prior to 1935 produced death with ulcers in 23 of 26 dogs in from 3 to 59 days, or in an average of 14.5 days. All had a gastritis.

One hundred mg per kilo body weight of cinchophen A and B was fed daily to 16 and 14 dogs respectively. The same quantity of cinchophen A and B was fed daily to 16 and 8 dogs respectively after a Rammstedt operation had been performed.

Results. The results are shown in Table II. It is evident that cinchophen A, the "impure" product, produced death with ulcer more

---

[6] Reid and Ivy, PROC. SOC. EXP. BIOL. AND MED., 1936, **34**, 142.

TABLE II.
Effect of Rammstedt Operation on Incidence of Ulcer Produced by Cinchophen.

Product	No. of dogs used	No. with ulcer	No. with gastric erosions	Normal sacrificed after 90 days	Avg survival time in days
Control.					
Cinchophen A, an "impure" product	14	12	2	0	21.5
Cinchophen B, a "pure" product	16	12	3	1	35
Total	30	24	5	1	
Rammstedt Operation.					
Cinchophen A	16	7	0	9	48.4
Cinchophen B	8	4	0	4	64
Total	24	11	0	13	

rapidly than cinchophen B. It is also clear that the Rammstedt operation reduced the incidence of ulcer by almost one-half. In the control group the incidence of ulcer was 80% (24 of 30 developed ulcer), and of ulcer and hemorrhagic erosions 96%. In the Rammstedt group the incidence of ulcer was 46% (11 of 24 developed ulcer) and of ulcer and hemorrhagic erosions 46%. A marked difference in the general condition between the control and Rammstedt animals was apparent, which was especially true of those Rammstedt animals that did not develop ulcer. These latter animals appeared to be healthy and only occasionally manifested anorrhexia and emesis, and their weight loss was slight. This was apparently due to the absence of gastritis revealed by autopsy.

Observations were made in regard to the location of the ulcer. In all the control animals, approximately 10% of the ulcers were located in the duodenum, 70% at, or in contact with, the pyloric ring, and the remainder along the lesser curvature. In the Rammstedt animals no duodenal ulcers were observed and none in relation to the pyloric ring. They were located along the lesser curvature near the incisura and were of the chronic, penetrating and cicatricial type, rather than of the acute, hemorrhagic and perforating type observed in the controls.

Conclusion. The Rammstedt operation decreases the incidence of gastritis, hemorrhagic erosions and ulceration that follows cinchophen administration in dogs. This indicates that pylorospasm plays a significant but not an all inclusive rôle in the genesis of cinchophen ulcers.

11585

Distribution of Ingested Phosphorus in Bone and Teeth of a Dog, Shown by Radioactive Isotope.*

MARIAN LEFEVRE MANLY, HAROLD C. HODGE AND STANLEY N. VAN VOORHIS.

From the Departments of Biochemistry and Pharmacology, School of Medicine and Dentistry, and the Department of Physics, The University of Rochester, Rochester, N. Y.

The penetration of ingested phosphorus into the enamel of cat's teeth has recently been reported by Hevesy and Armstrong.[1] These workers found that the enamel contained up to 10% of the radioactive phosphorus present in the dentine. Armstrong (personal communication) indicated that an important part of the radioactive phosphorus in the enamel might be adsorbed on the outer surface. One of us (M. L. M.) has recently shown that enamel can adsorb phosphates.[2] Before the report of Hevesy and Armstrong appeared, we had attempted to follow the distribution of P*† in a dog. The day before any P* was administered, the dog was anesthetized with nembutal. Grooves exposing or penetrating the dentine were cut with a small dental burr in each lower cuspid and in the upper right first molar to offer local stimuli for calcification. The P* (10 microcuries) was prepared in a solution containing 0.09 g of total phosphorus as Na_2HPO_4 and was given by stomach tube to an 8.8 kg dog in 4 approximately equal doses 2 to 6 hours apart on 2 successive days. The dog was sacrificed by bleeding under anesthesia 12 days after the last dose of P*. Bone samples were taken, the teeth were removed, enamel and dentine separated, and the organic matter extracted. The P* content of the various samples was determined using the Geiger-Müller scale-of-four counter. All counts on tissue samples were calculated to a 100% basis from the decay curve of a P* standard solution. The counts were corrected for total concentrations of calcium phosphate dissolved in each sample.

In Table I are given data for the P* distribution in the various bones examined. The percentage of the P* dose found in 1 g of the various tissues ranges from 0.002 in the diaphysis of the ulna to 0.10 in the spongy portion of the diaphyses of the humerus. In a

---

* This work was supported in part by grants from the Carnegie Corporation of New York and the Rockefeller Foundation.

[1] Hevesy, G., and Armstrong, W. D., *Sci. Proc. A. Soc. Biol. Chem.*, 1940, xliv.

[2] Manly, M. F., and Levy, S., *J. Am. Chem. Soc.*, 1939, **61**, 2588.

† P* = radioactive phosphorus.

TABLE I.
Distribution of Marked Phosphorus in Various Bones.

Tissue	Portion	Sample wt, g	% dose per g tissue
Ulna	diaphysis	.5595	.0024
Radius	”	1.0142	.0031
	epiphysis	.6248	.0059
	dense	.1722	.0021
	spongy	.0630	.0075
Humerus	dense diaphysis	.5945	.0039
	spongy ”	.0906	.10
	” ”	.1969	.075
Scapula	whole bone	.3331	.0121
	dense	.3684	.0086
	spongy	.0953	.057

TABLE II.
Distribution of Marked Phosphorus in Dentine.

Tooth		Wt of sample, g	% dose per g inorganic
Incisor	UL 2	.1906	.0026
”	UL 3	.3543	.0017
”	LR 3	.1529	.0026
Cuspid	LL	.1378	.0024
	LL	.7949	.0033
	UL	.3216	.0029
	UL	1.1926	.0026
	LR	1.2024	.0026
Bicuspid	LR 2	.0466	.0034
	LL 2	.1279	.0027
	LR 3	.2207	.0035
	UL 3	.2795	.0032
	LL 3	.2342	.0039
	LL 4	.2919	.0015
Molar	UL 1	1.1543	.0029
	UL 1	.5012	.0026
	LR 1	1.4079	.0024
	LL 1	1.4393	.0024
	UL 2	.6533	.0027
	LR 2	.1607	.0033
	LL 2	.1871	.0029
	LR 3	.1940	.0034
	UL 4	.1833	.0024

given bone, the dense portion has from 1/3 to 1/5 the P* content of the spongy portion.

In Table II are given the data on the P* content of the dentine samples. The percentage of the dose of P* found per gram of inorganic matter varies from 0.0015 to 0.0039. The principal characteristic of the distribution is the constancy from tooth to tooth; there is no tendency for incisor dentine to contain more or less P* than molar dentine. The amount of P* in the dentine is approximately the same as that found in the dense diaphyseal bone. This is in accord with the concept that dentinal mineral metabolism is similar to that of cortical bone but differs in degree of activity.[3] The 2 scarred teeth were split in such a way as to isolate the part containing the grooves and estimations were made both on this and the remainder of the tooth. There was no difference between the P* contents of the scarred and the unscarred parts. Within the 12-day experimental period, there was evidently no increase of phosphate turnover or deposition in response to the stimulus of scarring.

Several attempts were made to find evidence of the presence of P* in the enamel. In no case was a count obtained which was more than 0.2 or 0.3 counts per minute above the background count. This difference is either insignificant, showing no P* to be present, or indicates that the enamel contained less than 0.001% of the original P* dose. The disagreement with the findings of Hevesy and Armstrong may be due to the larger doses used by these workers. In a private communication, Armstrong indicated that with similar sized doses of P* he had found questionable evidence of P* in cat enamel.

Summary. The uptake of radioactive phosphorus was greater in spongy than in dense diaphyseal bone when salts of the element were administered orally to a dog. In dentine the amounts of radioactive phosphorus were constant for the various teeth and of the same order as that present in dense diaphyseal bone. No more than traces of P* were found in the enamel.

11586

Influence of Androgen Therapy on Growth Rate of Hypogonadal Adolescent Boys.*

BRUCE WEBSTER AND WALTER HOSKINS.†

From the Barbara Henry Research Laboratory, Department of Medicine, New York Hospital, and Cornell University Medical School, New York City.

The fact that the sex hormones play a rôle in the control of linear growth of the adolescent human has been previously suggested.[1] The exact relationship of the androgen-estrogen levels or the balance which exists between them, to the growth processes has not been understood. Experiments which suggest that the administration of androgens to the immature rat closes the epiphyses and retards growth have been reported.[2, 3] We were unable to confirm these findings in the immature rat and rabbit. McCullagh and McGurl[4] have reported epiphyseal closure in 2 cases of eunuchoidism treated with moderately large doses of androgens over a period of several months. On the other hand, Moricard and Bize[5] have reported an acceleration of statural growth in 3 adolescent hypogonadal boys treated with testosterone propionate at weekly intervals for 3 to 6 months. No control periods were reported. A calculation of the growth rate reported by these authors shows it to be 2.0 to 2.6 cm per hundred days. Villaret[6] reported a growth of 4 cm in a 21-year-old hypogonadal man during 6 months' treatment with large doses of testosterone propionate. The rate of growth here was 2.2 cm per hundred days. This patient had shown no increase in height since his eighteenth year when he had grown 9 cm "sous l'influence de l'opotherapie thymique qui fait descendre les testicles a l'anneau." Although these reports are apparently in conflict, numerous authors have expressed caution in administering androgen therapy for fear of closing the epiphyses and arresting growth.

---

[3] Manly, R. S., Hodge, H. C., and Manly, M. L., *J. Biol. Chem.*, 1940, **134**, 293.

* We are indebted to the Ciba Pharmaceutical Products Company, Lafayette Park, Summit, New Jersey, for the testosterone propionate used in the treatment of these cases.

† General Education Board Fellow, 1939. Barbara Henry Research Fellow, 1939-40.

[1] Webster, B., *J. Pediatrics*, 1939, **14**, 684.

Webster[7] reported a series of cases of hypogonadism in the adolescent male treated with testosterone propionate. No special mention of linear growth was made at that time, although weight-height observations were made on all the patients. Many of these patients had been under observation for several years before treatment was begun. All of them have been followed since that time. Following the publication of the above report, other cases of adolescent hypogonadism were treated by the same method. As treatment progressed considerable attention was given to the question of linear growth and epiphyseal closure, since it was feared that epiphyses might be closed and growth stopped by the sudden production of the sexual changes associated with puberty. After a survey of the results, however, it became apparent that a reverse effect was taking place. The growth rate was markedly increased during

---

[2] Rubinstein, H. S., Kurland, A. A., and Goodwin, M., *Endocrinology*, 1939, **25**, 724.

[3] Levie, L. L., *Acta brev. Neerland*, 1938, **8**, 53.

[4] McCullagh, E. P., and McGurl, F. J., *Endocrinology*, 1940, **26**, 377.

[5] Moricard, M. P., and Bize, P. R., *Société de Pediatrié*, 1937, **35**, 26.

[6] Villaret, M., Justin Besancon, L., and Rubens-Duval, A., *Compt. rend. Soc. de biol.*, 1938, **127**, 599.

[7] Webster, B., *J. Pediatrics*, 1938, **13**, 847.

periods of androgen therapy and X-rays of the knees, ankles, and wrists, taken at the beginning and end of therapy, showed no evidence of epiphyseal closure.

Each of the 8 cases presented in this report was considered to have definite hypogonadism. The effect of the therapy on this condition has been reported elsewhere.[7] They ranged in age between 13 and 18 years. All were in good health except for the evidences of hypogonadism. One case, No. 3, exhibited marked undernutrition during the control periods but gained .6 kg during the first 4 weeks of therapy. All of the remaining cases were in a healthy nutritional state. The measurements were taken under standard conditions with outer clothing removed. The dosage of testosterone propionate ranged between 75 and 125 mg per week. Each individual dose consisted of 25 mg.

The accompanying table shows the growth rate of 8 cases, ranging in age between 9 and 18 years, before, during, and after testosterone propionate therapy. In 2 instances a second period of treatment has been included.

The average growth rate of this group of hypogonadal adolescent boys before treatment with testosterone propionate was 1.36 cm per 100 days. During the administration of from 75 to 125 mg of testosterone propionate weekly, the average growth rate increased to 3.6 cm per 100 days. Following the cessation of treatment this rate

TABLE I.
Growth Rate of Hypogonadal Adolescent Boys During Treatment with Testosterone Propionate.
(cm/100 days)

Case	Age range, years	Total change in height, cm	Growth rate before treatment.		Growth rate during treatment.		Growth rate after treatment.		Growth rate during 2nd period of treatment.	
			cm	days	cm	days	cm	days	cm	days
1	14⅓-16	142-154	3.0	288	4.5	105	1.5	91	3.0	111
			1.0		4.3		1.6		2.7	
2	13½-16	160-176	2.8	150	8.2	250	5.0	340		
			1.9		3.3		1.5			
3	12¼-15	144-172	12.5	703	10.5	316	5.5	364		
			1.8		3.3		1.5			
4	9¾-15½	130-162	16.7	1339	9.7	381	5.6	408		
			1.2		2.5		1.4			
5	13¾-15½	152-170	0.3	33	2.4	66	6.8	275		
			1.0		3.6		2.3			
6	13-14	140-150			5.7	123	3.8	270		
					4.6		1.4			
7	11-14	145-161	10.8	802	4.2	196	1.0	77		
			1.3		2.1		1.3			
8	16½-18	164-176			5.8	120	6.0	391	1.2	56
					4.8		1.5		2.1	

again fell to 1.56 cm per 100 days. In 2 cases a second period of therapy again increased the average growth rate to 2.4 cm per 100 days. As was stated above, there was no evidence of hastening of epiphyseal closure as demonstrated by X-ray before and after the periods of administration of the testosterone propionate.

The evidence in favor of the possibility of the increased androgen or estrogen level of the body at puberty playing a rôle in epiphyseal closure and body growth has been steadily increasing during recent years. The exact nature of that rôle is as yet not clear. Contrary to expectations, the administration of androgens to a series of hypogonadal boys apparently stimulated longitudinal growth. This is compatible with Kenyon's and associates'[8] observation that there is a reduction in the urinary excretion of inorganic phosphorus, potassium and creatine, as well as protein retention, during androgen therapy. The stimulation of growth by the administration of thyroid substance has long been recognized.[1] The mechanism is thought to act through the thyrotropic factor of the anterior lobe of the pituitary. It would seem that a similar mechanism has been involved in the growth stimulation by androgen therapy and that a disturbance of the gonadotropic factor in some way reacts upon the growth factor. Whether or not this mechanism will work in the case of normal adolescents is not known since it is not considered advisable to administer androgens to normal individuals.

Summary. The administration of testosterone propionate in doses of 75 to 125 mg weekly to 8 hypogonadal adolescent boys ranging in age between 9 and 18 years was accompanied by an increase in average growth rate from 1.36 cm per 100 days during the control period to 3.6 cm per 100 days during the period of therapy. Following the cessation of treatment the average growth rate again fell to 1.56 cm per 100 days. This apparent stimulation of growth rate was unaccompanied by X-ray evidence of epiphyseal closure. The exact mechanism of this growth stimulation is not clear. Since all of these individuals exhibited evidence of hypogonadism, it must be pointed out that, on the basis of these observations, there is no justification for the use of testosterone propionate as a growth stimulant in normal adolescence since such therapy might result in permanent damage to the individual.

---

[8] Kenyon, A. T., Knowlton, K., Sandiford, I., Koch, F. C., and Lotwin, G., *Endocrinology*, 1940, **26**, 26.

11587 P

Studies on Typhus Rickettsiae Cultivated in Yolk Sac of Developing Chick.

K. H. Pang and Samuel H. Zia.

From the Department of Bacteriology and Immunology, Peiping Union Medical College, Peiping.

Although Barykine, *et al.,*[1] first reported the use of the yolk sac as a new culture medium for the classical type of typhus rickettsiae in 1936, little attention was paid to this method until Cox[2] showed conclusively that egg yolk is indeed an excellent medium for the growth of all types of rickettsiae. Since that time, a great deal of interest has been aroused in the practical application of this method, particularly for the production of an immunizing agent.[3, 4, 5] However, it appears to us that in order to make full use of this valuable procedure, a better understanding of the characteristics of rickettsiae so cultivated is desirable. In view of this, the results of preliminary studies carried out with local murine typhus strains are hereby reported.

The simple technic originally employed is followed in principle. Special attention was paid to the localization of the rickettsiae in the yolk sac, the successful cultivation from the blood of infected animals, and to the virulence of the organisms so cultivated for the white mice.

1. *Localization of the rickettsiae:* In spite of the statements made by previous workers[2, 6] that no intracellular organisms were found, it seems to us more reasonable to expect typhus rickettsiae to grow intracellularly in the yolk sac as in other culture media. By examinations of smear of the suspension of yolk sac cells, which float on the surface on brief centrifugation rickettsiae can invariably be demonstrated inside the cells in positive cultures. It is true that some extracellular organisms can also at times be found but they can be regarded as having been liberated from cells. This is in agreement with the recent findings of Rake and his co-workers[7] in their cultivation experiments with virus of lymphogranuloma inguinale.

---

[1] Barykine, W., *et al., Proc. 2nd Internat. Congress Microbiology,* 1936, 322; *Bull. Office Internat. Hyg. Publ.,* 1938, **30**, 326.

[2] Cox, H. R., *U. S. Pub. Health Rep.,* 1938, **53**, 2241.

[3] Cox, H. R., *U. S. Pub. Health Rep.,* 1939, **54**, 1070.

[4] Otto, R., and Wohlrab, R., *Z. f. Hyg. Infekt.,* 1939, **122**, 220.

[5] Zinsser, H., Plotz, H., and Enders, J. F., *Science,* 1940, **91**, 51.

[6] Rake, G., *et al.,* Proc. Soc. Exp. Biol. and Med., 1940, **43**, 332.

[7] Tchang, J., and Mathews, G. B., *Chinese Med. J.,* 1940, **57**, 47.

2. *Isolation of typhus rickettsiae from the blood:* As the yolk sac affords a suitable medium for massive proliferation of rickettsiae it was considered of interest to see if the less heavily infected blood of typhus guinea pigs would, as in the case of more heavily infected blood of spotted fever animals,[2] initiate successful cultures. As the hemoglobin of the cells would color the contents of the egg, and seems to cause the death of the embryo, the plasma has been used with some success. In 8 trials 4 successful cultures were obtained. It is apparent that the small number of typhus rickettsiae present in the circulating blood of infected animals could initiate cultures in a certain percentage of cases.

3. *Virulence of the rickettsiae cultured in yolk sac for white mice:* Although occasional strains of murine typhus have been found to be pathogenic for white mice[8, 9] the experience in general seems to indicate that normal white mice are resistant to the inoculation of infected materials ordinarily employed and an inapparent infection was the rule. In the present investigation, both freshly isolated and passage murine strains were employed. The latter strains, "S" and "N", have not produced any demonstrable infection in white mice during several years' observation in this laboratory. However, after one or more passages in the egg yolk sac, 20 out of 28 mice inoculated with "S" rickettsiae, and 6 out of 9 with "N" succumbed to the infection. The incubation period varied from 3 to 7 days and numerous intra- and extracellular rickettsiae were variably found in the peritoneal exudate. The identity of the typhus nature was made by negative culture on ordinary laboratory media and severe infection on reinoculation into guinea pigs with the peritoneal exudates. Thus it can be conclusively stated that murine typhus rickettsiae, after brief cultivation in the yolk sac, acquire a virulence, not only for the susceptible guinea pig,[10] but also for the naturally resistant white mice.

Comment and Summary. By paying special attention to a few fundamental characteristics, significant findings were noted which may have important practical implications. The concentration of the organisms in the cells of the yolk sac which can easily be separated by centrifugation would suggest a simple method for the removal of much undesirable inert materials contained in the yolk sac during the preparation of vaccine. The presence of these substances has been the main objection for employing this vaccine in human prophylaxis. Successful initiation of cultures from the blood of in-

---

[8] Okamoto, Y., *Kita. Arch. Exp. Med.*, 1937, **14**, 99.

[9] Giraud, P., and Panthier, R., *Bull. Soc. Path. Exot.*, 1939, **32**, 404.

[10] Parker, R. R., personal communication.

fected animals naturally leads to its possible application for diagnosis in patients, when test animals usually employed such as guinea pigs and white rats were not available. Preliminary studies in this connection have offered some indication as to its practical use. The fact that death is regularly produced in white mice by the yolk sac cultivated rickettsiae gives us an additional and convenient animal which may be employed for typhus studies. By its use, immunological and chemotherapeutic studies can be facilitated and extended.

11588

Urinary Excretion of Vi-Phage and *B. typhosus* Following Phage-Inoculation in a Typhoid Carrier.

C. H. YEN AND C. Y. CHEN. (Introduced by C. E. Lim.)

From the Departments of Bacteriology and Surgery, Peiping Union Medical College, Peiping.

Following the finding of Vi-phage specific for V form of *B. typhosus,* the influence of phagotherapy on typhoid infections has gained new interest. Asheshov, Wilson and Topley[1] have demonstrated the protective effect of a Vi-phage on experimental typhoid infection in mice when the phage was given not later than 4 hours after infection. In the same experiment a non-specific phage, which could lyse both V and W forms *in vitro,* was found to be ineffective. Later, Fisk[2] also showed the protective action of a typhoid phage on experimental typhoid infection in mice. He, however, did not indicate whether the phage employed was a Vi-phage or not. In view of the lack of information on the influence of Vi-phage in human infection it is considered desirable, in this communication, to present some of our observations made on the treatment of a case of a urinary carrier of *B. typhosus* with Vi-phage and non-specific phages.

The patient (CCL), male, aged 42, with a history of symptoms of pyelitis, cystitis, and urethritis for at least 18 years, was first seen 3 years ago when *B. typhosus* was detected in his urine. Roentgenological examination then suggested the presence of bilateral renal calculi. In the succeeding 3 years, the patient was given vigorous local treatment with various urinary antiseptics such as argyrol, and oral medication with sulfanilamide and mandelic acid, but the symptoms persisted and a type A V-form *B. typhosus,* as determined by the method of Craigie and Yen[3] was persistently isolated from the urine. During the same period the stool was repeatedly negative for *B. typhosus.*

---

[1] Asheshov, I. N., Wilson, J., and Topley, W. W. C., *Lancet,* 1937, **1**, 319.

[2] Fisk, R. T., PROC. SOC. EXP. BIOL. AND MED., 1939, **38**, 659.

The course of phagotherapy consisted of one subcutaneous injection of 1 cc of a combined phage followed by intramuscular injections of 2 cc of the same preparation at intervals of 2 to 3 days for 6 doses. The combined phage is a mixture of equal volumes of a potent type 2 Vi-phage of Craigie and Yen[3] specifically propagated on a type A V-form organism (P. 243) previously isolated from the patient's urine, and a non-specific "W"*-phage which lyses *in vitro* both V- and W-forms of *B. typhosus.* One cc of the combined phage contains 2.5×10^9 plaque units† of the Vi-phage and 3.5×10^8 plaque units of the "W"-phage. In addition to the phages, sodium bicarbonate was administered *per os* to maintain the alkalinity of the urine.

From 2 days before the first to 21 days following the last phage-injection, the urine and stool wehe repeatedly examined for the presence of antityphoid phages and *B. typhosus.* The stool consistently failed to show the presence either of antityphoid phage or *B. typhosus.* For studies of the urine, portions of non-catherized morning specimens were heated to 56°C for ½ hour immediately after collection and tested on plates previously flooded with susceptible organisms and freshly dried. For cultural studies the unheated portions of the same urines were inoculated on China-blue rosalic-acid medium. While it was impossible to ascertain that all the suspicious colonies obtained in these plates were *B. typhosus,* detailed examinations of a few of these from each specimen of urine examined have shown them to be *B. typhosus* as determined by fermentative and agglutinative tests. The results of daily excretion of Vi-phage and colony-counts of bacteria in the urine are summarized in Chart 1.

Results. The non-specific "W"-phage was not detected in any specimen of urine collected either before or after phagotherapy. This conspicuous failure to recover the "W"-phage from urine is interesting in the light of the lack of protective action *in vivo* of a non-specific phage reported by Asheshov, Wilson and Topley.[1] From the chart it can be seen that the Vi-phage began to appear in the urine in considerable quantity (1.4×10^5 plaques per cc) on the 7th-9th day;

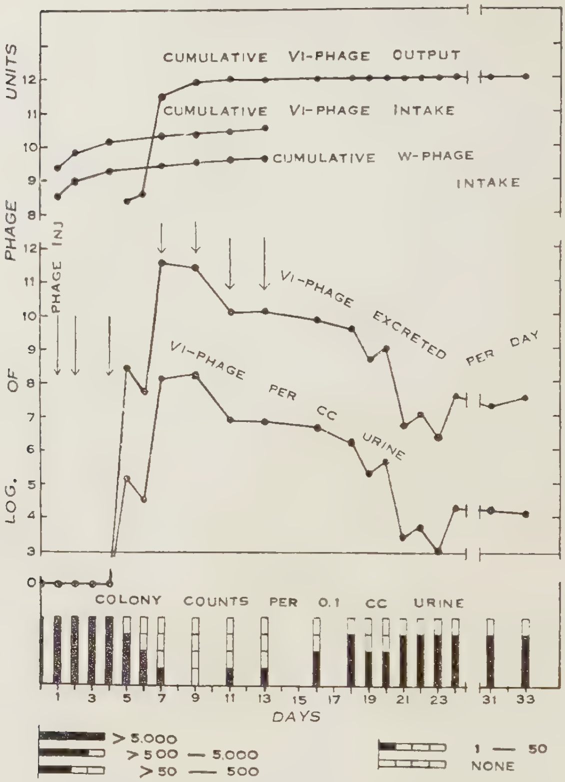
---

[3] Craigie, J., and Yen, C. H., *Canad. Pub. Health J.*, 1938, **29**, 448.

* The "W"-phage was isolated by one of us (C. H. Y.) from a lysogenic typhoid culture in 1937 at Dr. Craigie's Laboratory, Toronto. This phage also causes lysis of *B. paratyphosus A, B, C; B. choleræ suis,* and *B. aertrycke.*

† In this communication the units of phage are expressed in terms of the number of discrete plaques seen on agar-surface growths.

CHART 1 PHAGE AND BACTERIA EXCRETION



it then decreased significantly despite the fact that 2 more doses of phage were given and finally was maintained at a lower level (1.1×10^3-2.2×10^4 plaques per cc) for as long as 21 days after the last injection, at which time this observation was terminated. From the cumulative output and intake of phage and its daily excretion, it is evident that the Vi-phage must have multiplied within the body. Accompanying the first appearance of the Vi-phage in the urine there was a decided decrease in the colony-counts, and at the time when the output of Vi-phage was maximal there was actually a failure to obtain growth from the urine. Subsequently, as the concentration of Vi-phage in the urine became reduced, the colony-count again tended to increase, and from the 16th day onwards *B. typhosus,* while distinctly less frequent than before the treatment with phage, was persistently present. Coincident with the simultaneous presence of Vi-phage and V-form organisms in the urine, there appeared Vi-phage-resistant W forms and tiny colonies of a lysogenic form in the same specimens. These latter forms were not seen in the urine before the appearance of Vi-phage. The Vi-phage-susceptible V-forms isolated either before or after the appearance of the phage in the urine consistently belonged to the bacteriophagic type A of Craigie and Yen,[3] but were frequently non-motile. The motility, however, could be regained after a few subculturings in broth. The V-form organisms isolated from phage-containing urine were just as susceptible to the action of the same specimen of urine as to the action of the original Vi-phage. The Vi-phage isolated from the urine was specifically neutralized by the anti-Vi-phage type 2 rabbit serum and behaved like a ϕ A-preparation of Craigie and Yen in its differential action on the standard type-strains. Thus the phage recovered from the urine is identical with the Vi-phage administered.

The simultaneous existence in the virus of Vi-phage-susceptible V-form organisms together with the Vi-phage is difficult to explain. When a fresh specimen of phage-containing urine (1.1×10^3 plaques per cc) was incubated at 37°C overnight the Vi-phage-concentration was found to increase by at least 10^5 fold and the resultant cultures isolated from this were Vi-phage-resistant W-forms which were still susceptible to "W"-phage. When the incubation was made anaërobically *in vacuo* the multiplication of phage was only 10 to 10^2 fold and the Vi-phage-susceptible V-forms could still be recovered from the urine. This suggests that the absence of free air in the urinary bladder may be a factor favoring the incomplete action of Vi-phage on V-form organisms. The intervals between two successive voidings were next investigated in order to see whether a longer interval would allow a more complete action of the phage on the organism within body. Table I gives one such determination. It will be seen that both phage- and colony-counts varied greatly on different specimens of urine passed within a period of 24 hours. There appears to be no simple correlation between the duration of time the urine was retained in the bladder and the amount of phage and bacteria excreted. But the highest phage-count associated with a

TABLE I.

Variation of Phage and Bacteria Counts in Different Specimens of Urine Collected within 24 Hours.

Date	Time	Time interval between 2 voidings		pH	Phage units per cc	Bacterial colony per 0.1 cc
April 21, 1940	7:40 A.M.	5 hr	5 min	8.4	1.3×10^8	5.0×10
”	9:45 ”	2	5	8.4	8.3×10^7	1.0×10^2
”	1:50 P.M.	4	5	8.2	4.3×10^7	3.9×10^2
”	3:30 ”	1	40	8.6	9.7×10^3	1.3×10^3
”	8:40 ”	5	10	7.8	1.3×10^7	5.4×10^2
22	1:00 A.M.	4	20	7.0	2.6×10^4	9.0×10^2
”	7:05 ”	6	5	7.6	2.2×10^4	8.7×10^2

low colony-count was seen when the urine was passed after an interval of over 5 hours, whereas the lowest phage-count associated with a high colony-count was seen in the urine passed after an interval of only 1½ hours. This lack of straightforward correlation may be due to the presence of other factors such as the variation in the number of bacteria discharged, probably intermittently, into the urine from the local lesion. However, neither the pH value, which varied from 6.8 to 8.6; the specific gravity, which varied from 1.015 to 1.021; nor the daily output of urine, which varied from 1.5 to 2.2 liters, showed any correlation with the amount of phage on the number of bacteria voided.

The blood of the patient examined on the 16th day of phagotherapy, revealed the presence of potent neutralizing antibodies against both the type 2 Vi-phage and the "W"-phage. The agglutinins of the same serum were as follows: O-1:320; H-1:640; Vi-1:40. Control serum from a typhoid convalescent (possessing agglutinins O-1:640; H-1:1280, and Vi-1:60) was found to contain no phage-neutralizing power. Thus the development of antiphage in the patient's blood appears to be unrelated to the presence of agglutinins.

The existence of phage-antibodies and the other data presented above seem to offer a plausible explanation for the simultaneous existence of Vi-phage and V-form organisms in excreted urine, if one postulates the existence of a focus of infection in the urinary system as seems more than likely to have been present in this case. In the focal lesion, the V-form organisms may have, being protected from the action of phage by the serum, and phage-susceptible organisms discharged intermittently into the bladder-urine where the Vi-phage could bring about lysis. The completeness of lysis would then depend upon the duration of contact, and relative concentration of phage and bacteria. The lytic action probably does not progress with maximal rapidity on account of lack of free air. The excreted urine would contain, therefore, not only the Vi-phage-resistant W-forms and lysogenic V-forms resulting from the action of Vi-phage on V-forms, but also the susceptible V-forms which are intermittently discharged from the lesion and have not been acted upon by the Vi-phage. The presence in the bladder of a small amount of urine after each voiding would serve to supply the Vi-phage after phage-injections were stopped. In this way a state may be produced similar to that observed, namely, the simultaneous presence of Vi-phage and Vi-phage-susceptible organisms in the same specimen of urine over a long period of time.

Summary. Following subcutaneous and intramuscular infections of a Vi-phage and a non-specific antityphoid phage in a urinary typhoid carrier, only the Vi-phage appeared in the urine in amounts greater than those administered. The numbers of *B. typhosus* in the urine were transiently decreased as the result of phage-treatment but increased again considerably with the appearance of W-form and lysogenic V-forms in addition to the typical V-forms which had been persistently present. The presence of W- and lysogenic V-forms was attributed to the action of V-phage in the bladder-urine and the presence of the V-form organisms due to the intermittent discharge of organisms from a focus in which the action of the Vi-phage was probably inhibited. At the same time specific antibodies against both phages were developed in the blood. The type-specificity of the phage and bacteriophagic typing of the Vi-form organisms repeatedly isolated from the urine remained constant.

11589

Studies on Stability of Dilute Purified Tuberculins.

Sam C. Wong and George Ouyang.

From the Departments of Bacteriology and Immunology and Public Health, Peiping Union Medical College, Peiping.

Concentrated tuberculin is remarkably stable whereas diluted tuberculin usually undergoes rapid deterioration. By the use of alkaline buffer solutions as diluting agents, however, old tuberculin has been claimed to maintain its potency for 4 to 6 months.[1, 2, 3] On the other hand, when the same diluents were applied to purified tuberculins by the same authors less favorable results were obtained; deterioration of the product generally occurring in 4 to 6 weeks. It is evident that a suitable diluent has not been found. In the present report a phosphate buffer of pH 7.0 is described which in our hands has been found to maintain the potency of dilute purified tuberculins for at least 4 to 6 months.

Materials and method. The preparation of the purified tuberculin employed for the study was similar in principle to that of Seibert's PPD. Its potency and other properties will be reported elsewhere. For the present it is sufficient to state that comparative tests made on

---

[1] Douglas, S. R., and Hartley, P., *Tubercle,* 1934, **16**, 105.

[2] Gottschall, R., and Bunney, W. E., *J. Immunol.,* 1938, **34**, 103.

[3] Jensen, K. A., Bindslev, G., Moller, S., Hansen, A., and Lind, P., *Tubercle,* 1938, **19**, 385.

several hundred human subjects between our product and the PPD kindly supplied to us by Dr. Florence Seibert revealed no difference in activity. Only the first dose of the Mantoux test consisting of 0.00002 mg in 0.1 cc was used as a measurement of biological activity. It was thought that the result obtained with this strength would be representative of higher concentrations. The purified tuberculin was diluted with buffered saline, the method of preparation of which was as follows: To each liter of normal saline is added 20 cc of a stock buffer solution consisting of 62.5 g of $Na_2HPO_4.12H_2O$ and 14.4 g of $NaH_2PO_4.2H_2O$ in 500 cc of distilled water. The buffered saline has a pH of 7.0 and is sterilized by autoclaving at 122°C for 20 minutes. After cooling, 40 cc of 5% phenol is finally added to effect a 0.5% concentration. Purified tuberculins were accurately measured, diluted in this buffered saline and distributed in 20 cc bottles fitted with rubber caps. One set was kept at room temperature (20°C) and the other in the refrigerator (8°C). Testings were done entirely on young subjects between the age of 9 to 13 years by intracutaneous injections made on the dorsal surface of the forearm. Readings were made 48 hours afterwards. When comparative tests were made, one injection was given in each arm in approximately the same position. A set of syringes and needles was kept entirely for this purpose. Final testings (data presented in Tables I and II) were done with new syringes and needles. For comparison we used freshly prepared solutions of Seibert's PPD. The dilute tuberculin kept at room temperature was tested weekly for the first month and every other week thereafter on a group of 13 volunteers. Only one injection was given at each testing. The tuberculin kept at the lower temperature was tested at longer intervals.

Dilute tuberculins kept at 20°C. The nature of the reaction from week to week of individuals repeatedly tested remained almost constant. The results of tests made at the end of 4 months of the same subjects and 17 new ones are presented in Table I. Parenthetically it may be stated that the stability of the dilute tuberculin kept at room temperature greatly exceeded our expectation. The experiment was concluded at this time owing to the exhaustion of our testing material. From the data it is clear that the agreement between the dilute purified tuberculin and the freshly prepared PPD of Seibert's as a whole is close and the slight differences recorded are well within experimental errors. It is true that one subject was missed by the old material. On the other hand, 2 were also missed by the new material. Unfortunately, these subjects could not be retested. From our experience this irregularity is not uncommon among young ado-

TABLE I.

Comparison of Sizes of Reaction Between Freshly Prepared PPD and Diluted Purified Tuberculin kept at 20°C for 4 Months.

Name Tuberculin	Repeatedly Tested Subjects. LCH	FWH	HPY	TK	FPC	CMJ	LPC	SCK	SCL	CJK	WH	CHJ	HCC
†Purified	25*	17	10	14	15	7	10	16	15	10	12	12	8
‡Seibert's	23	13	8	14	15	7	22	18	16	15	14	12	12

Name Tuberculin	LSC	KSC	SWY	MTS	PWT	New Subjects. CSC	KCL	KFH	CH	LCF	CCC	CCT	CMH	LCM	LHM	TYS	MCC
†Purified	10	10	7	5	3	8	13	10	0	3	8	8	9	13	0	0	0
‡Seibert's	5	6	0	3	0	15	15	12	10	10	10	10	9	13	0	0	0

TABLE II.

Comparison of Sizes of Reaction Between Freshly Prepared PPD and Diluted Purified Tuberculin Kept at 8°C for 6 Months.

Name Tuberculin	HCL	TCK	HS	TYC	SCL	WHC	WC	CHC	CHH	CYF	KF	WWJ	CYA	CPT	LPC	WHY	KKY	LHH	LCH	16 subjects
†Purified	25	20	20	15	15	7	22	22	20	13	12	10	12	11	10	5	7	7	0	0
‡Seibert's	22	16	12	10	13	5	30	25	25	20	20	20	15	15	12	12	10	10	10	0

*All figures are in millimeters and represent diameters of reactions.
†Kept at temperature and time indicated.
‡Freshly prepared PPD.

lescents. Usually, however, retesting would correct these differences. Incidental observation was also made on Seibert's PPD kept at room temperature for 3 months. It was found that the reactions were similar to those elicited by the fresh preparation and thus indicated no deterioration.

Dilute tuberculin kept at 8°C. This was tested at the end of 3 and 6 months. Since the first result showed complete agreement with a fresh preparation of Seibert's PPD in 8 parallel tests it is necessary only to present the data obtained at the latter date. The results given in Table II show the comparative sizes of the reactions between the purified tuberculin and the PPD among 35 tested individuals. It can be seen from the table that there is a general agreement but the freshly prepared PPD of Seibert's elicited a stronger reaction in a larger number of positive reactors. However, the stability of the dilute purified tuberculin kept for 6 months at 8°C seems to be well established.

Comment. From the results presented above, 3 points are of interest. (1) Repeated endermal injections of human subjects over a period of 4 months with 0.00002 mg of our purified tuberculin each time, as expected, did not alter the sensitivity of the skin. This is shown by the remarkable constant reactions observed in the same individuals so tested. In other words it indicates that our purified tuberculin in the doses employed is not sensitizing. (2) The general impression that dilute purified tuberculin undergoes rapid attenuation even in buffer solutions is not borne out by the present study. Indeed we have yet to observe either marked or complete destruction of its activity preserved in a neutral buffer when it was kept at 20°C for 4 months and 8°C for 6 months. Why the buffer solution employed by us has a better preservative effect for purified tuberculins than those employed by others is not clear. It is possible that a neutral pH is an important factor although we have, as yet, no data to support our contention. (3) The observation made by Jensen, *et al.*,[3] that phenol inactivates dilute tuberculin is not substantiated by our finding since the preservative employed in our buffer is phenol. Because the study is made on only 2 preparations (one of ours and one of Seibert's) it is premature to apply this conclusion to purified tuberculin in general. Still we believe the simplicity of the buffer and the preservative effect so far shown have much to recommend it for further trials in the field of purified tuberculin.

Summary. A phosphate buffer is described which has been found to maintain the skin-reactivity of dilute purified tuberculin for at least 4 months at 20°C and 6 months at 8°C.

11590

Experimental Streptococcal Infections in Animals for Therapeutic Investigations. I. Virulence-tests.

C. H. Huang and R. H. P. Sia

From the Department of Medicine, Peiping Union Medical College, Peiping.

The recent introduction of sulfanilamide and sulfapyridine in the treatment of *Streptococcus hemolyticus* infections has aroused a great deal of interest. It was felt that a further study of the effect of the drug in experimental hemolytic streptococcal infections produced in animals would be highly desirable. It is recognized that experimental work on *Streptococcus hemolyticus* has been handicapped by the lack of a susceptible laboratory animal. Although there are a few virulent strains which can kill mice at a dilution of 10^{-6} or 10^{-7} when injected intraäbdominally, their value is somewhat limited for several reasons. (1) As Kolmer[1] has pointed out a highly virulent strain produces fatal infection too rapidly for therapeutic investigations, since the severity of the infection usually does not permit the administration of repeated doses of the agent under study and may mask any slight curative activity which is insufficient to produce a complete disinfection in one or 2 doses at daily intervals. (2) A drug which works well in the virulent strains may not do the same in all strains of *Streptococcus hemolyticus* infections. (3) It is highly desirable to compare the virulence of the organisms isolated from different patients. At the outset of our study it was found that the strains of hemolytic streptococci obtained locally from human sources were relatively avirulent for laboratory animals. It was necessary to employ a relatively large dose, namely, 0.1 to 0.05 cc of an 18-hour serum-broth culture, to kill a mouse when injected intraäbdominally. A few attempts made to increase the virulence of some of our strains of hemolytic streptococci by repeated passages through mice were not successful. This finding rendered our proposed study difficult, since protection-tests conducted with large infecting doses of organisms are in general not very satisfactory.

Kolmer[1] introduced the endermal infection in rabbit and observed the rate of healing of the lesion for the therapeutic investigation. This requires the use of a large animal and a moderately virulent strain *i.e.* a strain that will kill a mouse with 0.001 cc of an 18-hour broth culture when given intraäbdominally. The strains isolated locally were not so virulent. Miller[2] was able to enhance the virulence of meningococcus for mice by the use of mucin. A similar procedure tried by us with hemolytic streptococci has so far yielded negative results. Norton and Dingle[3] have recently shown that intracerebral injections of *E. typhi* in mice resulted in an increase in the lethal effect of the organism for the animal when compared with intraäbdominal injections. This method was tried with several strains of hemolytic streptococci isolated recently from our cases of scarlet fever and erysipelas and was found to be satisfactory. So the aim of this study is to compare the lethal effects of *Streptococcus hemolyticus* for small laboratory animals when the organisms are introduced intraäbdominally, interpleurally, intravenously, and intracerebrally.

---

[1] Kolmer, J. A., and Rule, A. M., *J. Lab. Clin. Med.*, 1937, **22**, 1097.

Experiments. Because of the shortage of white mice at the time when this study was started, Chinese hamsters of 15-20 g were employed at first. Two strains of *Streptococcus hemolyticus* were used, namely, SA_{17} and E_1 which were isolated recently from patients suffering from scarlet fever and erysipelas respectively. The amount given intraäbdominally and intrapleurally was 0.5 cc of the desired dilution of the organism, while that given by the intracerebral route was 0.05 cc. Animals receiving interpleural or intracerebral injections were previously anesthetized with ether. The organism used was an 18-hour culture in a 5% rabbit-serum, 0.1% dextrose, meat-infusion broth. For each route of inoculation 5 different dilutions of organisms were given and for each dilution 3 animals were used. Animals were kept for 3 weeks before they were discarded. The result is shown in Table 1. All the animals were necropsied after death. Direct smears and cultures were made from the places of inoculation and also from the heart blood. In all animals the direct smears and cultures from the places of inoculation were positive for *Streptococcus hemolyticus.* Cultures from the heart blood, however, gave positive results in about 30% of the animals.

Similar experiment was repeated with SA_{17} strain on hamsters with the same result.

Another experiment was carried out on white mice instead of hamsters with strains SA_{17} and 232. In this experiment, the inter-

---

[2] Miller, C. P., *Science,* 1933, **78**, 340.

[3] Norton, J. F., and Dingle, J. H., *Am. J. Pub. Health,* 1935, **25**, 609.

TABLE I.

Comparative Lethal Doses of *Streptococcus hemolyticus* for Hamsters.

No. of organism injected	SA_{17} E_1	4 x 10^7 25 x 10^6	4 x 10^6 25 x 10^5	4 x 10^5 25 x 10^4	4 x 10^4 25 x 10^3	4 x 10^3 25 x 10^2	4 x 10^2 250	40 25	4
Route									
Intraäbdominal	SA_{17}	*1, 1, 1	1, 1, 2	S,S,S	S,S,S	S,S,S	—	—	—
	E_1	1, 1, 1	1, S,S	S,S,S	S,S,S	S,S,S	—	—	—
Interpleural	SA_{17}	—	1, 2, 6	1,17,S	S,S,S	S,S,S	S,S,S	—	—
	E_1	—	1, 1, 1	1, 1, 1	1, S,S	S,S,S	S,S,S	—	—
Intracerebral	SA_{17}	—	—	—	—	1, 1, 1	1, 1, 2	2, 3,S	1, S, S
	E_1	—	—	—	1, 1, 1	1, 1, 1	1, 2, 2	1, 2,S	1, S, S

*Each numeral indicates days of survival of the animal after the infection.
S = Animal remained well 21 days.

TABLE II.

Comparative Lethal Doses of *Streptococcus hemolyticus* for White Mice.

No. of organism injected									
	SA_{17}	3 x 10^8	3 x 10^7	3 x 10^6	3 x 10^5	3 x 10^4	1,800	180	18
	232 1.P	12 x 10^7	12 x 10^6	12 x 10^5	12 x 10^4	12 x 10^3			
	1.C					7,000	700	70	7
	1.V	23 x 10^6	23 x 10^5	23 x 10^4	23 x 10^3				
Route									
Intraäbdominal	SA_{17}	*1, 1, 1	1, 1, 1	3,S,S	S,S,S	S,S,S	—	—	—
	232	1, 1, 2	2, 2, 6	14,S,S	S,S,S	S,S,S	—	—	—
Intracerebral	SA_{17}	—	—	—	—	—	1, 1, 2	2, 6, 5	1,S,S
	232	—	—	—	—	1, 1, 1	1, 2, 2	2,S,S	1,S,S
Intravenous	232	1, 1, 1	1, 1, 2	3,S,S	S,S,S	—	—	—	—

*Each numeral indicates days of survival of the animal after the infection.
S = Mouse remained well 21 days.

pleural injection was omitted. The amount given intracerebrally was 0.03 cc instead of 0.05 cc. With strain 232, the intravenous route was added and the amount given by this route was 0.1 cc. The result shown in Table II, is just the same as those found in hamsters.

Inoculation by the intracerebral route with strain 232 was repeated 3 months after the first isolation. It was found that the virulence of the organism was markedly decreased.

Studies on the virulence of different strains and different types of *beta Streptococcus hemolyticus* isolated from patients with different diseases and from normal individuals will be reported later.

Conclusion. Hamsters and white mice are suitable animals for testing virulence of *Streptococcus hemolyticus* when injected by the intracerebral route. It requires only 40-400 organisms to kill the animal when given intracerebrally, whereas it requires 400,000 when given interpleurally and 4,000,000-11,500,000 when given intraäbdominally and intravenously.

11591

Effect of Vitamin C on Creatine and Creatinine Metabolism.

CH'UAN FAN AND THERESA T. WOO. (Introduced by Fu-tang Chu.)

From the Division of Pediatrics, Department of Medicine, Peiping Union Medical College, Peking, China.

During a study of creatine and creatinine metabolism in hypothyroidism by one of us,[1] one of the experimental subjects was utilized for studies on vitamin C[2]. It was noted that when the patient was saturated with the vitamin, there was a perceptible increase in the creatine and creatinine excretion. This is shown in Fig. I. Further, the daily fluctuation in the subsequent period seemed to be augmented. We, therefore, repeated the experiment on 4 normal children and on a case of glycogen disease in which the tolerance to creatine has been demonstrated to be much decreased.[3]

Experimental. The subjects were admitted to the metabolic ward

---

[1] Fan, Chuan, Creatine and creatinine metabolism in hypothyroidism, in preparation.

[2] Chu, F. T., and Sung, C., *Chinese Med. J.*, 1937, **52**, 791.

[3] Fan, Chuan, and Woo, Theresa T., *Chinese Med. J.*, Pediatric Number, 1940.

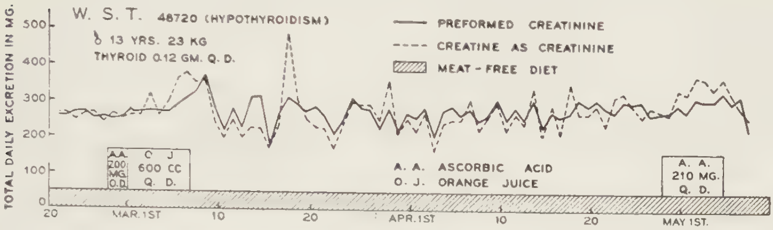


FIG 1

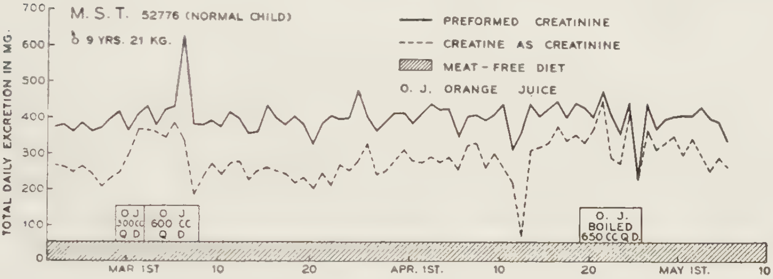


FIG. 2

where the preparation of the diet and the collection of the urine are carried out under careful supervision. They were all put on a constant diet adequate in proteins and caloric intake. Except in the case of H.E.C.T., meat was not allowed during the period of study. At the end of each daily period, the subjects were instructed to empty their bladders completely. The urine specimens were kept on ice with toluol as preservative. Analysis was done, with few exceptions, within 24 hours by the method of Folin.[4] Creatine was dehydrated by autoclaving with picric acid purified by crystallization as sodium picrate.[5] Acetone in urine does not interfere with the determination by this method.[5] We have also satisfied ourselves that ascorbic acid added to the urine specimens does not introduce any difficulty in the analysis.

Results and Discussion: The results of the study are charted in Fig. I to VI. It is evident that in the 4 normal children, no definite increase of either creatine or creatinine could be demonstrated even though the curves are suggestive. There was also a suggestive increase in the fluctuation in the daily excretion of these substances in the subsequent periods. If vitamin C did increase creatine formation in the body, such increase must be small so that the creatinuria was not significantly increased. In order to confirm this suspicion, we need a subject whose tolerance to creatine must be minimal. Such a subject we had in the case of glycogen disease.[3]

---

[4] Folin, O., *J. Biol. Chem.*, 1914, **17**, 469.

[5] Peters, J. P., and Van Slyke, D. D., *Quantitative Clinical Chemistry*, 1932, Vol. II, pp. 597 and 600.

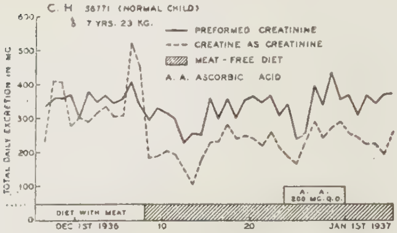


FIG. 3

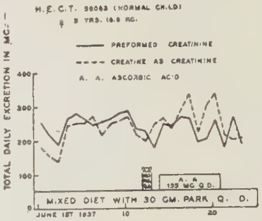


FIG. 4

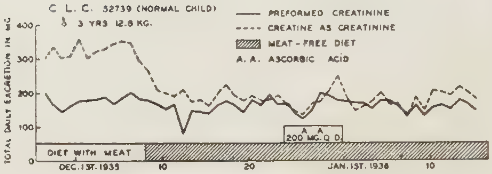


FIG. 5

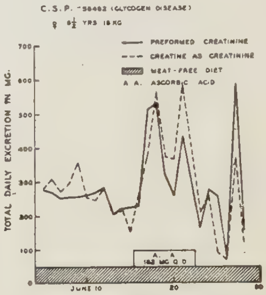


FIG. 6

Indeed when we look at Fig. VI, we see a markedly different picture. On the administration of ascorbic acid both creatine and creatinine in the urine showed remarkable increase, possibly due to an increase in creatine formation in the body. That vitamin C may have such an action is also suggested by the work of Palladin and Epelbaum[6] who reported decrease in creatine in skeletal muscles in severe scurvy. Whether this action is connected with the role of vitamin C in tissue oxidation is not known.

Summary. In a case of glycogen disease, whose tolerance to creatine is known to be minimal, saturation with vitamin C caused a marked increase in the urinary excretion of creatine and creatinine. In 4 normal children and in a child with hypothyroidism, however, no significant increase was demonstrated. This is probably due to the fact that whatever increase in creatine formation due to the ingestion of ascorbic acid is covered by the normal capacity of storage. The mechanism of the action of vitamin C on creatine metabolism is not understood.

---

[6] Palladin, A., and Epelbaum, S., *Biochem. Z.*, 1929, **204**, 140.

11592 P

Rhythmic Precipitation of Pneumococcal Soluble Specific Substances and Antipneumococcal Sera.

RACHEL BROWN

From the Division of Laboratories and Research, New York State Department of Health, Albany.

In order to extend the observations on pneumococcal antigen-antibody reactions *in vitro,* precipitations were allowed to proceed slowly in a solid medium. The resulting rhythmic phenomenon resembles in many respects that noted by Liesegang when crystalloids react in a gel.[1] Other workers (Bechhold,[2] Petrie,[3] Kirkbride and Cohen,[4] and Reiner and Kopp[5]) have noted this phenomenon also in serological reactions.

For the present experiments, antipneumococcal sera were mixed with 3% gelatin in physiological salt solution in tubes (11- by 75-mm), and the solution of antigen layered over the solidified gelatin. The tubes were corked and the reactions allowed to proceed for several weeks at refrigerator temperature. Eighteen types of unconcentrated and 4 types of concentrated antipneumococcal horse sera, as well as types 1 to 32 rabbit sera were used. Highly purified soluble specific substances,[6] pneumococcal "C" substance, and pneumococcal nucleoprotein were taken as antigens. Results were duplicated readily when conditions were carefully controlled. The concentration of both reagents and the age and concentration of the gel were important. Failure in any instance to obtain rhythmic precipitation may be due to improper experimental conditions.

There was great variation in the character of the results obtained when the specific carbohydrate solution was layered over the homologous serum-gelatin mixture. (Plate I) Series of closely packed, very fine and sharply defined rings, as well as series of broad and more or less indistinct bands, and intermediate types of reactions were observed. The pattern obtained varied with the type of pneu-

---

[1] Liesegang, R. E., *Naturwiss. Wchnschr.,* 1896, **11**, 353 (Cited in Freundlich, Herbert, *Colloid and Capillary Chemistry,* 3d ed., New York, Dutton & Co., 1922, p. 732.)

[2] Bechhold, H., *Z. f. physik. Chem.,* 1905, **52**, 185.

[3] Petrie, G. F., *Brit. J. Exp. Path.,* 1932, **13**, 380.

[4] Kirkbride, M. B., and Cohen, S. M., *Am. J. Hyg.,* 1934, **20**, 444.

[5] Reiner, L., and Kopp, H., *Kolloid-Z.,* 1927, **42**, 335 (Cited in *Chem. Abs.,* 1927, **21**, 3949.)

[6] Brown, Rachel, *J. Immunol.,* 1939, **37**, 445.

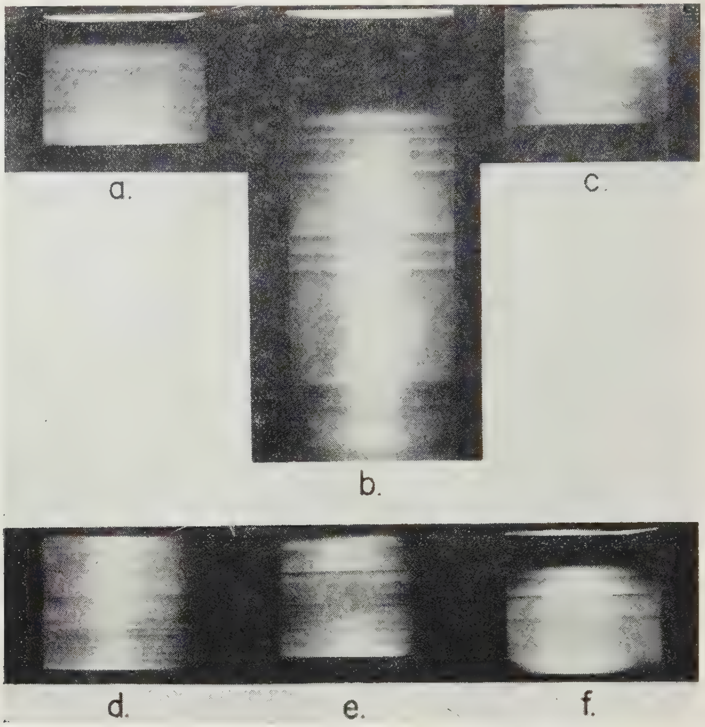


PLATE I.
Rhythmic precipitation exhibited by homologous soluble specific substance and antipneumococcal serum. *a.* Type 3 rabbit serum; *b.* Type 5 rabbit serum; *c.* Type 12 horse serum; *d.* Type 14 horse serum; *e.* Type 19 horse serum; *f.* Type 25 rabbit serum.

mococcus. Also, the character of the reaction was dependent on the individual serum or animal and on the preparation of soluble specific substance. In general, the reaction was more extensive in the tubes of horse serum than in those of rabbit serum. Particularly characteristic of the latter was a clear interval just below the contact surface and above a zone of precipitation. The extent of the reaction was not related to the viscosity of the antigen solution.

Certain immune horse sera exhibited a more complex pattern than others of the same type. This was especially true of all bleedings examined from a particular horse immunized against type-12 pneumococcus. (Plate I) Sera from this horse also reacted with pneumococcal "C" substance and with nucleoprotein. Sera of very low potency gave a continuous hazy reaction rather than the rhythmic reaction of more potent sera. By adjustment of the quan-

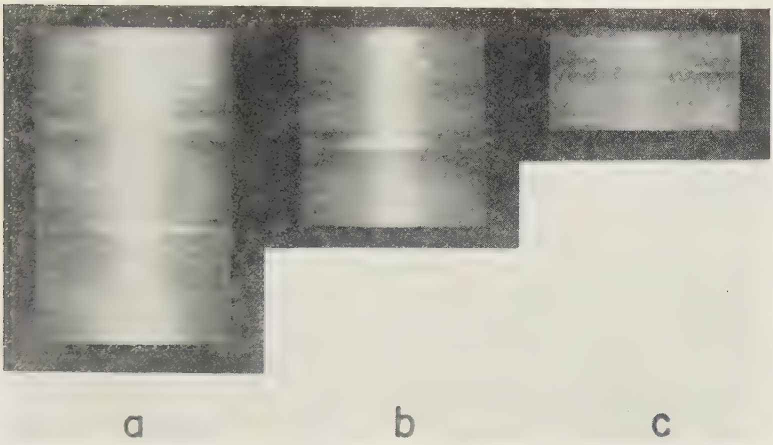


PLATE II.
Rhythmic precipitation exhibited by decreasing amounts of type 3 soluble specific substance and a constant amount of type 3 antipneumococcal horse serum. *a*. 1:400 dilution; *b*. 1:2000 dilution; *c*. 1:10,000 dilution.

tities of both reagents, a definite zone was determined in which maximal reaction occurred.

In one experiment, 3 type-2 horse antisera of different potencies according to the mouse-protection test, showed very similar results when the same number of units of each was used with the same optimal amount of soluble specific substance; but when equal volumes of these 3 sera were taken, marked variations were noted.

In another experiment with decreasing amounts of type-3 soluble specific substance and a constant amount of homologous horse antiserum, the pattern was similar but progressively less extensive. (Plate II)

Conclusion. These observations on the rhythmic precipitation of pneumococcal antigen-antibody reactions suggest that the phenomenon is comparable to that occurring with crystalloids in a gel.

11593

Metabolism of α,β- and β,γ-Deuterocaproic Acids.*

MARGARET G. MOREHOUSE AND H. J. DEUEL, JR.

From the Department of Biochemistry, University of Southern California Medical School, Los Angeles.

In earlier studies it has been demonstrated that deuterium may be retained on the betahydroxybutyrate molecule excreted after feeding deuterobutyric acid to rats.[1] When the α,β-dideutero acid was fed, only 4.2 atoms% were retained, while after feeding the β,γ-dideuterobutyric acid, an average of 22.5% of the excreted betahydroxybutyrate contained deuterium.

In order to determine which part of the caproic acid molecule is convertible to acetone bodies, a study of the deuterium content of betahydroxybutyric acid excreted in the urine after the feeding of the α,β- and β,γ-dideuterocaproate has been made. The deutero acids were prepared by saturation of the corresponding unsaturated acids.

The α,β unsaturated acid was formed by condensation of butyraldehyde and malonic acid under the influence of pyridine as a base while the formation of the β,γ hexenoic acid was a condensation of the same substances with triethanolamine as a base. They were identified by saponification numbers, boiling points, indices of refraction, and the rate of iodine uptake of the acids and their corresponding ethyl esters.

The acids were fed by stomach tube in the form of their sodium salts to female rats previously fasted 72 hours. The amounts administered were equivalent to 150 mg per 100 sq cm (calculated as acetone) per day in 2 divided doses. The urines of 8 animals were pooled, the betahydroxybutyric acid separated by acidification, extraction in butyl alcohol, and the subsequent extraction from this material as the sodium salt by means of sodium carbonate. The silver salt was then prepared. This was analyzed for betahydroxybutyric acid and its deuterium content. The results are shown in Table I.

A production of acetone bodies was found to occur after the feeding of the 2 dideuterocaproic acids. The amounts recovered on the fourth day of the fast as betahydroxybutyric acid varied from

---

* This work was made possible by a research grant from The Best Foods, Inc.

[1] Morehouse, M. G., *J. Biol. Chem.*, 1939, **129**, 769.

TABLE I.

Excretion of Deutero-β-hydroxybutyrate After Feeding Deuterocaproic Acids.

Exp. No.*	Amt of deutero-caproate fed as acetone	Acetone bodies excreted as acetone in mg			% of total as β-hydroxy-butyrate	β-hydroxy-butyrate excreted as such	Analysis of Ag β-hydroxybutyrate		% of caproate fed excreted as acetone
		β-hydroxy-butyrate	Diacetic	Total			% β-hydroxy-butyrate	Atoms % of deuterium	
				αβ-deuterocaproic acid.					
	mg					mg			
1-4	2928	1194	338	1532	77.9	2150	99.7	6.77	52.3
2-4	2844	1373	341	1714	80.1	2462	96.7	4.95	60.3
1-5	2928	1354	408	1762	76.9	2427	98.5	7.05	60.2
2-5	2844	1498	428	1926	77.8	2688	99.7	6.95	67.7
Avg					78.2		98.8	6.43	
				βγ-deuterocaproic acid.					
3-4	2877	1400	500	1900	73.7	2510	98.9	13.07	66.0
4-4	2855	1442	483	1925	74.9	2584	96.1	8.95	67.4
3-5	2877	1594	576	2170	73.4	2858	96.1	11.82	75.4
4-5	2855	1753	615	2368	74.0	3143	96.4	9.71	82.9
Avg					74.0		96.9	10.89	

*The second number represents the day of the fast.

†Calculated by the factor 9.51 for mg of mercury precipitate equivalent to 1 mg hydroxybutyrate (Blunden, H., Hallman, L., Morehouse, M. G., and Deuel, H. J., Jr., *J. Biol. Chem.*, 1940, **135**, 757).

52.2 to 66.7% of that fed. On the following day the recovery was higher varying from 60.1 to 82.7%. The animals fed the β,γ-dideutero acid excreted in all cases slightly higher proportions than did those which received the α,β compound.

The analyses of the silver salts of betahydroxybutyrate showed a sufficient purity that it would be impossible for the deuterium present to be in the form of a contaminant.

The analysis for deuterium indicates that in the case of the rats fed α,β-deuterocaproate an average of 6.43% of the betahydroxybutyric acid excreted contained deuterium (calculated on the basis of one atom to a molecule of acid). Those receiving the β,γ-caproate excreted a hydroxy acid which averaged 10.89% of the deutero-containing acid.

If the betahydroxybutyrate had originated from the α,β-deuterocaproate solely by beta oxidation, then no deuterium would be present because the β carbon atom containing the deuterium would be replaced by a carboxyl group. The retention of deuterium which did occur can best be explained on the basis of a delta oxidation. On the other hand the higher deuterium content occurring after feeding the β,γ-deuterocaproate would also seem to indicate that delta oxidation had played a part. Inasmuch as the deuterium is retained to a greater extent when on the γ carbon than on the α carbon atom in betahydroxybutyrate,[1] the higher retention here can be explained as partly traceable to delta oxidation with a consequent retention of the deuterium on the γ position. These experiments would seem to indicate the probability that delta oxidation plays an important role in the oxidation of caproic acid.

11594

Reaction of Dog's Colon to Subcutaneous Injections of Morphine.

R. D. TEMPLETON, E. A. GALAPEAUX, AND H. F. ADLER

From the Departments of Physiology, Loyola University School of Medicine, and the University of Chicago.

An increase in intestinal tone following the administration of morphine seems to be the most consistent result reported by various investigators. According to Plant and Miller[1] the most pronounced and lasting effect of morphine upon the colon of unanesthesized dogs and men is an increase in the muscular tone. Gruber, Greene, Drayer and Crawford[2] observed an increase in the tone of Thiery-Vella fistulae of dogs following the intravenous injection of morphine. These observations were confirmed by Krueger,[3] Gruber and Brundage,[4] Forster[5] and others. On the other hand, a few investigators have reported a decrease in intestinal tone following morphine administration. Gruber and Robinson[6] observed that repeated injections of morphine led to a general decrease in the tonus of the intestine. Krueger, Howes and Gay[7] have reported that small doses of morphine are followed by a decrease in intestinal tone while large doses caused an increase.

---

[1] Plant, O. H., and Miller, G. H., *J. Pharm. and Exp. Therap.*, 1927, **32**, 437.

Reports on the effect of morphine on intestinal activity have been reasonably consistent when dosage is taken into consideration. Plant and Miller[1] reported that peristaltic activity of the colon is increased by largely eliminating the periods of quiet while the time occupied in the passage of an individual wave was not materially changed. They also observed that the amplitude of peristaltic contractions varied inversely with the increase in tone. Krueger[3] reported a decrease in rhythmic frequency and an increase in peristalsis and tone. The number of peristaltic waves obtained after morphine, according to Krueger, Howes and Gay[7] increases with increasing doses and reaches a maximum when about 2 mg per kg are given, after which the number of waves becomes less. They further observed that the amplitude and frequency of rhythmic contractions were dependent upon the size of the dose administered, and that the frequency was inversely related to the amplitude. Puestow[8] reported from observations on human patients that morphine stimulated small bowel peristalsis but inhibited or paralyzed the right colon.

From a preliminary study on the motility of the colony of 2 dogs, Templeton and Galapeaux[9] reported that ¼ grain of morphine administered subcutaneously does not materially alter the normal activity 24 hours later even after 124 daily injections. They also reported that the immediate effect is an augmentation of activity which lasted for at least 200 minutes. This work has now been extended to include periodic injections of morphine in 2 additional dogs.

---

[2] Gruber, C. M., Greene, W. W., Drayer, C. S., and Crawford, W. M., *J. Pharm. and Exp. Therap.*, 1930, **38**, 389.

[3] Krueger, H., *J. Pharm. and Exp. Therap.*, 1934, **50**, 254.

[4] Gruber, C. M., and Brundage, J. T., *J. Pharm. and Exp. Therap.*, 1925, **53**, 120.

[5] Forster, A. C., PROC. SOC. EXP. BIOL. AND MED., 1938, **38**, 332.

[6] Gruber, C. M., and Robinson, P. I., *J. Pharm. and Exp. Therap.*, 1929, **37**, 101.

[7] Krueger, H., Howes, H., and Gay, H., *J. Pharm. and Exp. Therap.*, 1931, **55**, 288.

[8] Puestow, C. D., *Am. J. Physiol.*, 1938, **123**, 165.

[9] Templeton, R. D., and Galapeaux, E. A., *Am. J. Physiol.*, 1937, **119**, 414.

The 4 animals used in this study had been under laboratory conditions and used for other colon studies over a period of approximately 2 years. During the training period these animals were required to lie on a cushioned table of the same type and under similar environmental conditions that were to be used in the experimental studies. During this time no efforts were made to prevent ordinary noises and disturbances encountered in the laboratory.

Colon motility was studied by the tandem balloon[10] technic[11] previously described. This technic consisted of 2 tandem systems each composed of 3 balloons arranged in a linear order. The balloons which measured 3 cm at the largest diameter and 3 cm in length were spaced 3 cm apart.

The small tubes (3/32 inches in diameter) leading from the balloons were glued together and in the areas where the balloons were tied in place a rubber base was constructed in order to make each balloon an air tight, separate compartment. These tubes leading from the balloons were each connected to water manometers by way of a "T" tube.

For recording motility one set of balloons was inserted by way of the cecostomy into the proximal colon and the second set was inserted by way of the anus into the distal colon. The most proximal balloon was located just distal to the ileocolic sphincter and the most distal balloon just proximal to the internal anal sphincter. The systems were then guyed into place to prevent shifting of the balloons into other segments during the course of an experiment. In order to smooth out the contour of each balloon it was alternately inflated and deflated. Standard inflation was accomplished by drawing 6 cm of water pressure against each balloon. The deflated balloons were clamped out of the circuit and 8 cm of water pressure raised in each manometer. The clamps were then released between the balloons and manometers thus establishing a closed system for recording pressure changes in the balloons.

Certain difficulties are encountered in the study of colon motility which make variations in a planned routine unavoidable. Such factors as trauma from inserting balloons, slight variations in balloon distention, noises, time of feeding, posture of the animal, temperatures of the laboratory, sleep and admonishments make it necessary for the investigator to know the extent to which these experimental variations alter colon motility. After a careful study of experimental variations encountered in the course of an experiment, Templeton and Borkon[12] concluded that the technical variables are of minimal importance when reasonable efforts are made to control the experimental conditions, since more deviation from the usual routine relative to the above factors could be introduced into the procedures without producing a measurable effect.

---

[10] Templeton, R. D., and Bollens, W. F., *J. Lab. and Clin. Med.*, 1930, **15**, 585.

[11] Templeton, R. D., and Lawson, Hampden, *Am. J. Physiol.*, 1930, **96**, 667.

On the 4 trained animals used 34 control experiments were conducted for the purpose of determining the trend of colon activity to be expected over a 400 minute period. Of the animals used in this study 2 received daily subcutaneous injections of morphine. The other animals were given the same quantity of morphine at weekly intervals. The dosage selected was ¼ grain morphine sulphate which had been found in preliminary studies to produce only slight depression on the general condition of the animal. It was observed that usually from 2 to 15 minutes after morphine injections a significant increase in the heart beat and respiration occurred which was of short duration. With this quantity of morphine gastric disturbance seemed to be related to the individual animal. In one of the animals a slight reaction was manifest by slight salivation and a few retching movements. In another animal, vomiting was almost invariable, while in the other 2 these side actions were not apparent.

The usual methods of studying the effects of substances upon the motility of the gastrointestinal tract have been to grossly compare tracings before and after introducing the experimental condition, or to measure the height of manometer excursions and count the number of pressure changes within a given period. In order to obtain a numerical representation of activity which would more adequately express variations from time to time, this method described by Borkon and Templeton[13] has been followed. A diagrammatic representation of each tracing (Fig. 1) was first transferred to graph paper according to two arbitrary rules. First, the gut was considered active if a contraction or contractions continued for one minute or more. Second, the gut was considered quiet if 2 minutes or more intervened between contractions. By this procedure the active and quiet periods are represented in sharp contrast. The

---

[12] Templeton, R. D., and Borkon, E. L., *Am. J. Dig. Dis.*, 1939, **5**, 809.

[13] Borkon, E. L., and Templeton, R. D., *Am. J. Physiol.*, 1937, **118**, 775.

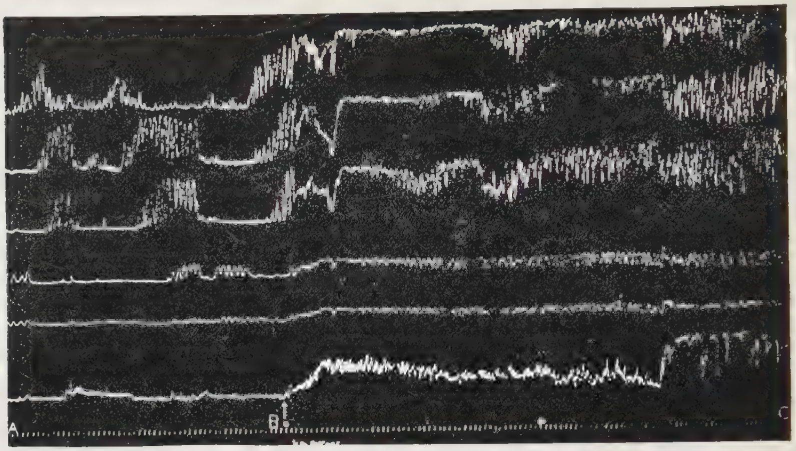
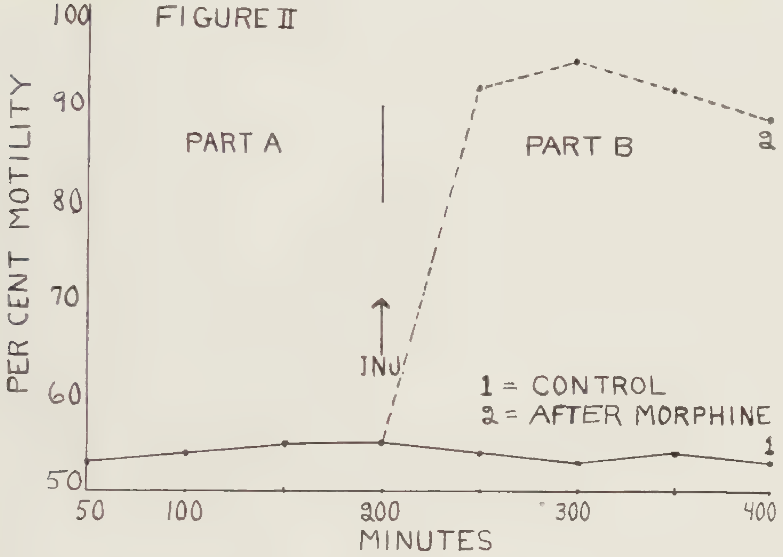


FIG. 1.
A-B—Control Colon Motility.
Arrow—Subcutaneous Injection ¼ Grain Morphine.

average quantity of activity per segment of the colon per 50 minute period throughout the course of the experiment was easily obtained from the diagrammatic charts by counting the number of minutes in which all segments were active and dividing the sum by the number of segments recorded. From these data the average per cent of activity per 50 minute period was computed.

In studying the influence of morphine on colon activity two factors must necessarily be considered: (1) the possibility of a cumulative effect which, if influencing colon motility, would be seen in the first 200 minutes of the tracings; and (2) the immediate effect of morphine which would appear in the period immediately following the injection. In considering the first factor the quantity of activity appearing in the colon after a number of daily injections was compared to the quantity of activity observed in the control tracings. The data for this comparison were obtained from the 200 minute control period which preceded morphine injections in the latter part of the study on the 2 animals which received daily injections, and the first 200 minutes of control tracings which preceded the morphine study on the same animals. This comparison revealed (Fig. 2) that the average percentage of activity per 50 minute period in eleven 200 minute tracings taken before the administration of morphine varied between 48 and 57%, while the percentage of activity obtained in 10 tracings after 60 daily injections of morphine and while still receiving daily injections varied between 50 and 60%. This small difference is probably of no significance since



it is well within experimental variation. Another approach to this question would be to compare the influence of prolonged daily injections upon the immediate effect of morphine. From this comparison it was observed that after 60 days of daily injections the average response obtained in 10 experiments was comparable to that which followed the first 10 injections.

From the above data it is apparent that the cumulative effect of morphine within the scope of our experimental time was of dubious importance. Therefore, the second factor, which deals with the immediate effect of morphine must account for the entire physiological disturbance to colon motility following its administration.

Data concerning the immediate effect of morphine on colon activity was obtained from all 4 dogs previously referred to, namely, the 2 animals which received daily injections and the 2 which received weekly injections. Some experiments were obtained on the 2 animals receiving weekly injections in which water was injected subcutaneously instead of morphine. These experiments were considered as controls for the morphine injections and included among the regular control tracings.

A total of 71 experiments were conducted in which the first 200 minutes of each constituted a control period. At the close of this control period ¼ grain morphine was injected subcutaneously in 37 experiments while 34 experiments were continued as controls either without injection or with subcutaneous injections of water. In the 200 minute control period the quantity of activity varied from

53 to 57%. In the experimental period the activity in the control experiments varied from 61 to 56%, while the activity of the morphine experiments varied from 89 to 94%. This great increase in activity continuing throughout the 200 minute period which followed morphine injections is characteristic of each experiment.

Summary. 1. The immediate effect of morphine on colon motility resulting from the subcutaneous injection of ¼ grain is essentially a marked increase in the tone, with a diminution, frequently a disappearance of quiet periods. 2. Alternating with the periods of high tone is an exaggeration of Type I components of Type II contractions. 3. As far as the colon motility is concerned the dog shows no increased tolerance to ¼ grain of morphine given daily. 4. No evidence of a cumulative effect on colon motility was seen to follow daily injections of ¼ grain of morphine for a period of 60 days. 5. Twenty hours after subcutaneous injection of ¼ grain morphine the colon motility is practically the same as that prior to the injection.

11595

Inhibitory Effect of Quaternary Ammonium Synthetic Detergent on Metabolism of Dental Plaque Material.*

Benjamin F. Miller, John A. Muntz and Sigmund Bradel.
(Introduced by G. F. Dick.)

From the Walter G. Zoller Memorial Dental Clinic and the Department of Medicine, University of Chicago.

The carious lesion begins almost invariably on the outer surface of the tooth, *i.e.* under plaque material, and works its way toward the interior. It appears likely that the metabolism of dental plaque material† is intimately associated with the pathogenesis of dental caries. Therefore, it would seem possible to prevent or retard the formation of early lesions if the metabolism of the plaque material could be inhibited completely, or even if it could be reduced to a subclinical level. This paper presents some experiments to that end.

---

* A preliminary report of these experiments was presented before the Subsection on Dentistry of the American Association for the Advancement of Science, Columbus, Ohio, December 30, 1939 (Abstracted in *J. Am. College of Dentists*, 1940, 7, 80).

† "Plaque material" is the thin film, composed of bacteria embedded in organic substance, which remains on the surface of the teeth after swabbing with cotton and washing with a stream of water.

It was shown previously by one of us[1] that experimental rat caries is inhibited markedly by the addition of fluoride or iodoacetate to the food and water. Presumably, these enzyme inhibitors interfere with the metabolic activities of the surface flora of the rat molars. However, the local or systemic toxicology of these compounds for man is not fully understood. Therefore, it has seemed desirable to look for compounds, capable of producing the same effects, which are less toxic and which possess the additional property of rapid penetration through the dense mass of human plaque material. The quaternary ammonium type of germicidal, wetting and detergent compound described by Domagh[2] appeared to possess these qualities. This compound, alkyldimethylbenzyl ammonium chloride,‡ reduces the surface tension of water even at very low concentrations, acts as a wetting and cleansing agent, and has relatively low toxicity for tissues.[3] Also, as shown by Miller, Baker and Harrison[4] it exhibits a remarkably potent inhibitory action on

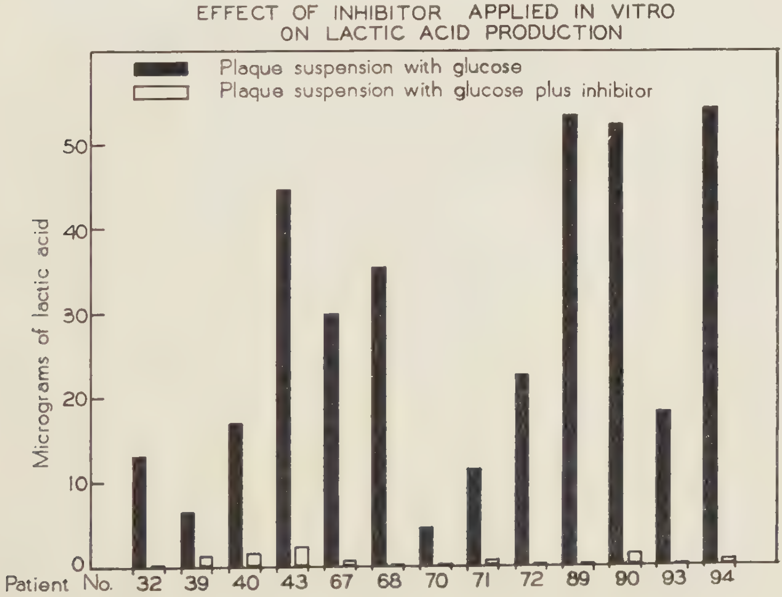


FIG. 1.

---

[1] Miller, B. F., PROC. SOC. EXP. BIOL. AND MED., 1938, **39**, 389.

[2] Domagh, G., *Deutsche Med. Wochenschr.*, 1935, **61**, 829.

‡ We are indebted to the Alba Pharmaceutical Co. for samples of this compound.

[3] Wright, L. T., and Wilkinson, R. S., *Am. J. Surg.*, 1939, **44**, 626.

[4] Miller, B. F., Baker, Z., and Harrison, R. W., PROC. SOC. EXP. BIOL. AND MED., 1939, **42**, 705.

the metabolism of pure cultures of microörganisms usually found in dental plaque material and lesions of early dental caries. These authors demonstrated that 2 to 25 μg of the compound inhibit the glycolysis or respiration of a billion bacterial cells within 5 to 10 minutes, and that the full effect could be obtained in dilutions as low as 1 part in 30,000.

In this paper, the action of alkyldimethylbenzyl ammonium chloride on the metabolism of the total plaque material is studied by *in vitro* and *in vivo* experiments.

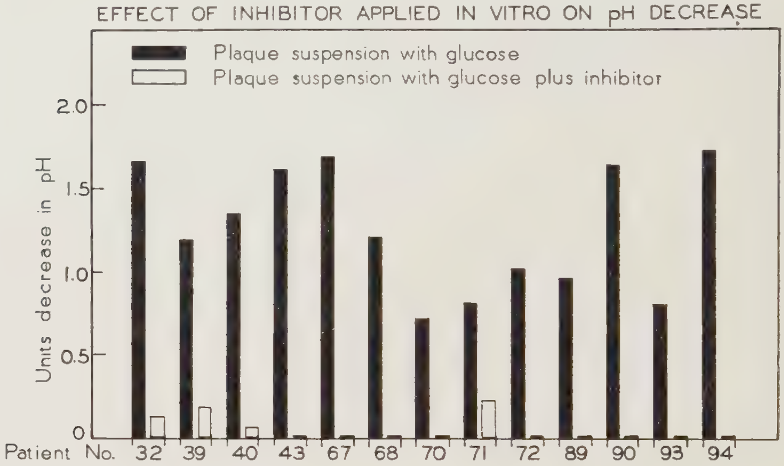


FIG. 2.

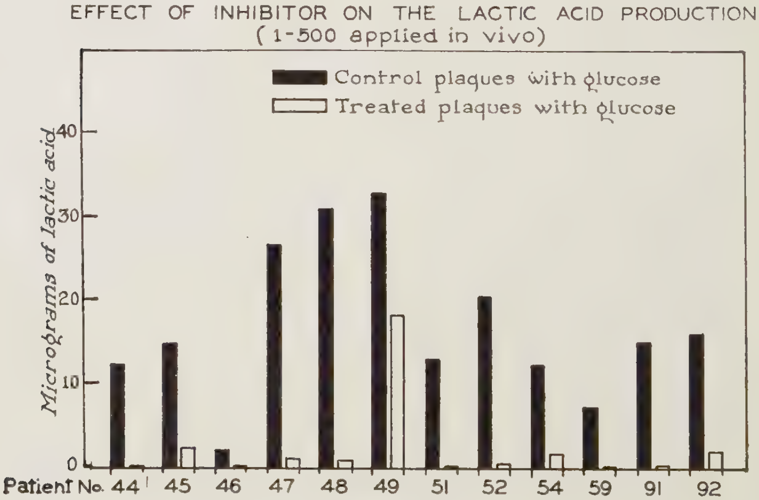


FIG. 3.

In view of the present state of our knowledge of dental caries, it must be emphasized that no definite conclusions concerning the prevention or treatment of the disease can be drawn from this report. The efficacy of any agent or procedure proposed for the cure, prevention or mitigation of dental caries can be determined only on the basis of results obtained from clinical studies.

Experimental. For the *in vitro* experiments, plaque material§ from 10 to 15 areas on the buccal surfaces of premolar and molar teeth was removed and homogenized in 0.6 to 0.8 cc of water. The plaque suspension was divided into 3 portions. Initial pH and lactic acid content were determined on one portion; the other 2 were incubated at 37°C for 30 minutes, one containing 0.5% glucose alone, and the other 0.5% glucose plus sufficient aqueous detergent to give a final concentration of one part in 3000 (approx. M/1000). Lactic acid was determined by the method of Miller and Muntz;[5] the pH drop was measured with a micro glass electrode. (It was demonstrated that neither determination was affected by the quantities of detergent employed.)

The lactic acid production, with and without the alkyldimethylbenzyl ammonium chloride, is shown in Fig. 1. It is apparent that this compound, in the concentration employed, suppresses very effectively aerobic glycolysis by the plaque material. Similarly, at the same concentration, it prevents any significant decrease in pH, as demonstrated in Fig. 2.

That alkyldimethylbenzyl ammonium chloride reacts with the plaque material very rapidly and tenaciously is demonstrated by the results of cases 89 and 90 in Fig. 1. In these experiments the compound was added to the homogenized plaque substance in the usual way; the suspension was centrifuged immediately, and the precipitated plaque material resuspended and incubated in fresh glucose solution which did not contain the inhibitor. It was found that the lactic acid production was completely suppressed.

In vivo application of alkyldimethylbenzyl ammonium chloride. In these experiments control plaque material was removed from 5 to 6 areas from one lower quadrant of the mouth, and incubated for 30 minutes in 0.5% glucose. The lactic acid and pH drop were determined as before. To corresponding areas on the other side of the subject's mouth, a 1:500 solution of alkyldimethylbenzyl ammonium chloride (approximately 0.006 molar) was applied with a cotton swab directly to the plaque areas for 2 minutes. When the swab was removed the subject was permitted to talk and salivate for 8 to 10 minutes. The treated plaques were then removed, placed immediately in 0.5 cc of the glucose solution, and incubated in the same manner as the untreated controls. The results in Fig. 3 demonstrate that the application of the detergent *in situ* reduced the capacity of the plaque material to produce lactic acid upon subsequent incubation in glucose solution. In 11 of the 12 cases the inhibition was essentially complete.

---

§ The plaque material was obtained from both caries-free and caries-active patients.

[5] Miller, B. F., and Muntz, J. A., *J. Biol. Chem.*, 1938, **126**, 413.

To ascertain the extent to which the alkyldimethylbenzyl ammonium chloride becomes fixed to the plaque material before removal of the plaques, the following experiment was performed: plaque substance was treated *in vivo* as above, removed and suspended uniformly in water, and immediately thereafter centrifugated at high speed. The precipitate was then resuspended in fresh glucose solution and incubated. The results, Nos. 91 and 92 in Fig. 3, show that the inhibition is as complete as in the other experiments. It was shown further that the supernatant fluid from the first suspension did not contain an inhibitory concentration of the detergent. This experiment demonstrates that the effect observed *in vivo* is extremely rapid, and is due apparently to a tenacious adsorption of the inhibitory compound on the plaque material.

Summary. Dental plaque material was suspended in glucose solution and incubated with the quaternary ammonium wetting and detergent compound, alkyldimethylbenzyl ammonium chloride. One part of this compound in 3,000 (approximately M/1000) completely inhibited the production of acid by the plaque material.

In another type of experiment, a 1:500 solution of the detergent was applied to the plaque material *in situ* for 2 minutes. The plaque material was removed 10 minutes later, and did not produce appreciable quantities of acid when it was then incubated in glucose solution.

11596

Effect of Atropine on the Local Action of Procaine.

A. J. Leser and C. H. Thienes.

From the Department of Pharmacology, University of Southern California.

Since the discovery of local anesthetics attempts have been made to obtain a prolongation of their action.

Modification of physical properties, such as tonicity and neutrality, forming of various salts, as well as increasing concentration does not lead to any satisfactory prolongation.[1, 2] Epinephrine has been recommended and is widely used; however it is generally recognized that this drug has a large range of contra-indications: in vasolabile patients, in patients with Graves' disease, and in surgery of very vascular areas (teeth, tonsils) with the dangers of quick resorption or accidental intravenous injection. It has also been shown that the addition of epinephrine to procaine injected intra-

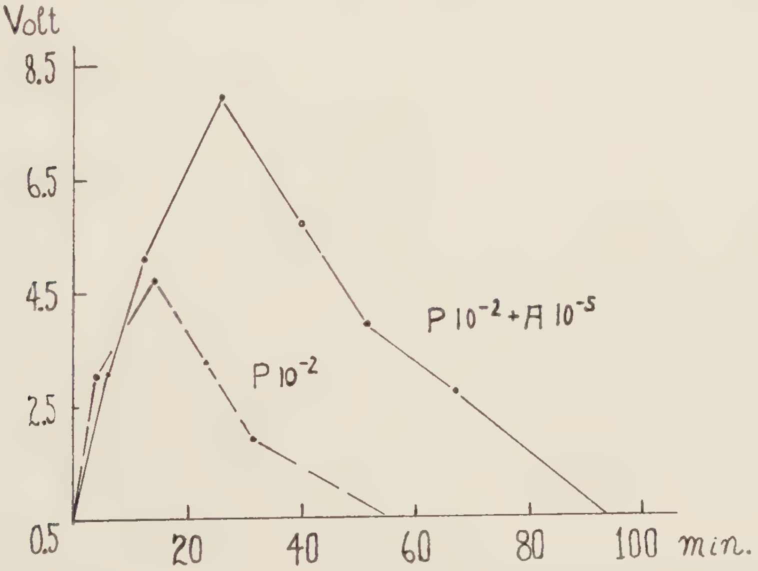


Fig. 1.

Anesthesia of the rabbit's cornea produced by Procaine HCl 1:100 without ($P\ 10^{-2}$) and with Atropine sulfate 1:100,000 ($P\ 10^{-2} + A\ 10^{-5}$).

Abscissa: Duration of anesthesia in minutes.

Ordinate: Least amount of electric stimulus in volts (from the secondary coil of the inductorium) producing wink reflex.

---

[1] Hirschfelder, A. D., and Bieter, R. N., *Physiol. Rev.*, 1932, **12**, 190.

[2] Leser, A. J., *J. Pharm. and Exp. Ther.*, 1940, **68**, 389.

venously into the guinea pig increases the toxicity of procaine 30-40 times.[3]

In an effort to replace epinephrine, addition of different drugs to procaine had been tried previously.[4] As it seemed possible that a parasympathetic depressant would exhibit an effect similar to that of a sympathetic stimulant (epinephrine) atropine was used in the further course of these experiments. The drug was added to a 1% solution of procaine HCl, which was then injected under the conjunctiva of a rabbit's eye. Corneal sensitivity was determined by means of an inductorium; the details of the test are described elsewhere.[2]

The graph shows the effect of atropine on procaine anesthesia. A slight increase in intensity and a very remarkable prolongation of anesthesia was obtained with concentrations of atropine sulfate as low as 10^{-5} (48 experiments). With these small amounts of atropine a diffuse bleaching of the conjunctiva was noted; dilatation of the pupil did not exceed the degree occasionally observed with procaine alone. Addition of atropine in higher concentrations 10^{-3} (12 experiments) resulted in a marked reddening of the conjunctiva and dilatation of the pupil, and showed no definite effect on intensity or duration of procaine anesthesia. It is possible that some systemic or vagal effects obtained with these higher concentrations interfere with the purely local action of atropine in low amounts.

The anesthetic action of atropine 10^{-5} has been found equal to about 10^{-3} of procaine HCl. This slight anesthetic effect is well known (Sollmann[5]) and may be correlated with the chemical relationship of atropine and cocaine as tropine derivatives. On the basis of pure summation the addition of atropine sulfate 10^{-5} to procaine HCl 10^{-2} would equal an increase of procaine concentration by 0.1%, which is known to give only very little prolongation.[6]

The effect of atropine sulfate 10^{-3} and 10^{-5} on the permeability of the frog's skin was tried. Small areas of skin of pithed frogs were cut out and left for 10-30 minutes in solutions of atropine 10^{-3} and 10^{-5} in frog-Ringer; controls from symmetrical areas were left in normal frog-Ringer. The membranes were mounted on small glass tubes with their surface outside. The tubes were immersed into a N/10 sodium thiocyanate solution and filled with ferric chloride in frog's Ringer as indicator. Temperature (20°C) and the amount of fluid used were kept constant. The degree of diffusion of thiocyanate ions through the membrane is indicated by the intensity of the color (red) in the tubes Although frog skins show a wide range of individual variation a careful comparison of symmetrical skin-areas seemed justified. Atropine 10^{-5} resulted in a decrease of permeability of the membrane for thiocyanate as indicated by the color test, while higher concentrations (10^{-3}) showed no definite effect. The same was true of the stain taken up by the membranes after 24 hours. These results suggest that prolongation of procaine anesthesia by atropine may be due to changes in permeability.

---

[3] Eichholtz, F., and Hoppe, G., *Arch. f. exp. Path. u. Pharm.*, 1933, **173**, 687.

[4] Leser, A. J., and Scholl, R., *Wien. klin. Wschr.*, 1937, **50**, 1577.

[5] Sollmann, T., *Manual of Pharmacology*, 5th ed., W. B. Saunders, 1936.

[6] Sinha, H. K., *J. Pharm. and Exp. Therap.*, 1936, **57**, 199.

Results obtained with other drugs of the atropine group, acetylcholine, as well as clinical trials will be reported elsewhere; it may be stated here that syntropan seems to be superior to atropine.

Summary. Prolongation of procaine HCl anesthesia by addition of atropine sulfate in low concentration 10^{-5} is reported; tests were made on rabbit's cornea after subconjunctival injection. The possibilities of an effect of low amounts of atropine sulfate on permeability are discussed.

Acknowledgment is due to Prof. A. J. Clark (University of Edinburgh) for suggestions and interest in the first stage of these experiments and to the Earl Moray Fund of the University of Edinburgh for financial aid.

11597

Sulfanilamide, Sulfapyridine and Sulfathiazole and Experimental Infections in Mice Due to *Shigella paradysenteriae* Flexner.

MERLIN L. COOPER AND HELEN M. KELLER. (Introduced by Glenn E. Cullen.)

From the Children's Hospital Research Foundation, and the Department of Pediatrics, College of Medicine, University of Cincinnati.

Our earlier studies[1] of acute diarrhea in 207 infants and young children revealed that 50% of the patients who were admitted to the Children's Hospital during the summer of 1938 with diarrhea had dysentery bacilli *(Shigella paradysenteriae)* in their stools. The majority of these patients were infected with the Flexner type, a few with the Sonne type. The mortality was 17%. With the advent of sulfanilamide and allied compounds and a search of the literature failing to find that such drugs had been used in experimental infections with Flexner type of dysentery bacilli, animal experiments were planned to determine the effectiveness of sulfanilamide, sulfapyridine and sulfathiazole.

---

[1] Cooper, M. L., Furcolow, M. L., Mitchell, A. G., and Cullen, G. E., *J. Pediat.*, 1939, **15**, 172.

The culture used had been isolated a year previously from the stool of an infant acutely ill with dysentery. Colony studies on MacConkey's medium revealed "rough" and "smooth" colonies. The "smooth" were avirulent in broth and in 3% mucin. The "rough" were slightly virulent in broth and quite virulent in 3% mucin. The virulence in mucin varied some, but a 20-hour Difco Brain Heart Infusion Broth culture diluted 1-100,000 in freshly prepared 3% autoclaved suspension of mucin killed the majority of white mice

TABLE I.
Therapeutic Effectiveness of Sulfanilamide, Sulfapyridine, and Sulfathiazole Administered in Diet. ½ cc of 1:100,000 culture in 3% mucin given intraperitoneally.

Mice	Diet plus 1% sulfathiazole ad lib. before culture	Diet plus 1% drug ad lib. after culture	Results
6	2 days	Sulfathiazole	All survived
6	3 "	"	" "
6	4 "	"	" "
6		Sulfanilamide	2 died
6		Sulfapyridine	6 "
6		Sulfathiazole	3 "
18			14 "

TABLE II.
Therapeutic Effectiveness of Sulfanilamide, Sulfapyridine and Sulfathiazole When Administered by Stomach Tube and in the Diet. ½ cc of 1:100,000 culture in 3% mucin given intraperitoneally.

Mice	Drug by stomach tube, 3, 6, 17, 27 hours after culture 4 doses*	mg	Results Died
6	Sulfanilamide	2	0
6	"	1	1
6	"	½	5
6	"	⅒	3
6	Sulfapyridine	2	0
6	"	1	0
6	"	½	0
6	"	⅒	5
6	Sulfathiazole	2	0
6	"	1	0
6	"	½	0
6	"	⅒	1
18			16

*Immediately after the last dose of drug by stomach tube the mice were placed on a diet, *ad libidum*, for 7 days containing 1% of the respective drug administered previously by stomach tube.

when injected intraäbdominally in ½ cc doses. The white mice weighed 17 to 20 g each.

Early in our experiments we found, as seen in Table I, that all mice were protected if given, *ad libidum,* a complete diet, to which had been added 1% of sulfathiazole, for 2 or more days before and continued for 7 days after injecting the organisms. The animals remained apparently normal. Table I shows also that sulfanilamide, sulfapyridine, and sulfathiazole were only partially effective therapeutically when administered in the diet *ad libidum after* injection of the culture. All the mice became sick within 5 hours after injecting the bacteria and since they ate very little food there was a low intake of drug.

In Table II is seen the therapeutic efficiency of surprisingly small doses of sulfanilamide, sulfapyridine, and sulfathiazole when given by stomach tube. All the mice survived in previous experiments in which 10 mg to 5 mg of these drugs were administered by stomach tube. All of 18 mice survived when treated with 2 mg of these drugs. Only one of 18 receiving 1 mg died, while 5 of 18 receiving 0.5 mg died, and 9 of 18 receiving 0.1 mg died. These mice had 4 doses of the drugs and were then given, *ad libidum,* the diet containing 1% of the respective drugs. Administration of the drugs by stomach tube apparently carried the majority of the mice through the early hours of acute infection, after which they ate sufficient food to insure adequate drug intake to obtain a therapeutic effect. All the mice were observed for a month and remained normal.

In the experiment set forth in Table III, one quantity, 2 mg of sulfanilamide, sulfapyridine, and sulfathiazole, was the standard dose throughout. The time elapsing between the injection of the organ-

TABLE III.

Effect of Delaying the First Dose of Drug on the Therapeutic Efficiency of Sulfanilamide, Sulfapyridine, and Sulfathiazole.

Mice	Culture 1:100,000 in 3% mucin	2 mg drug by stomach tube at indicated hours after the culture*	hr	Results Died
6	½ cc intraperit.	Sulfanilamide	*2*, 8, 22, 27	0
6	" "	"	*3*, 9, 22, 27	0
6	" "	"	*4*, 10, 22, 27	0
6	" "	"	*5*, 11, 23, 28	0
24	" "	Sulfapyridine	" " " "	0
24	" "	Sulfathiazole	" " " "	0
12	" "			9

* Immediately after the last dose of drug by stomach tube the mice were placed on a diet, *ad libidum,* containing 1% of the respective drug administered previously by stomach tube.

ism and the administration of the first dose of drug by stomach tube varied between 2 and 5 hours. This interval was without significance, however, since all the mice survived. All the mice were very sick within 5 hours after injecting the culture and the rapid favorable response to the drugs was marked. All the mice were observed for a month and remained well.

Blood level determinations on pooled blood from mice which had been on each of the 1% drug diets for 7 days gave the following data:

Sulfanilamide-free 5.8 mg % and conjugated 2.9 mg %
Sulfapyridine ” 7.3 ” ” ” ” 1.1 ” ”
Sulfathiazole ” 2.9 ” ” ” ” 0.5 ” ”

After this paper had been submitted for publication our attention was called to the work of Buttle,[2] who cured mice infected with the Sonne type of dysentery bacilli by administering 25 mg of sulfanilamide twice daily for 7 days. This worker used only Sonne cultures because he had “ . . . not yet obtained Shiga or Flexner strains of sufficient virulence to enable us to test the effect of the drug in these infections.”

Summary. All mice survived when fed, *ad libidum,* a complete diet containing 1% of sulfathiazole for 2 or more days before and 7 days after injecting *Shigella paradysenteriae* Flexner. Seven of 18 mice survived when offered, *ad libidum,* 1% drug diets of sulfanilamide, sulfathiazole, and sulfapyridine immediately after injecting the organisms. Fifty-seven of 72 mice survived when given 4 feedings by stomach tube of these 3 drugs in doses varying between 2 mg and 1/10 mg. All of 72 mice survived when given 4 feedings of 2 mg of these 3 drugs by stomach tube, regardless of whether the first dose of drug was administered 2, 3, 4, or 5 hours after the injection of the organisms. Nine of 48 control mice survived.

---

[2] Buttle, G. A. H., *Proc. Roy. Soc. Med.*, 1937, **31**, 154.

11598

Thermostability of Heat-Stabile Components of Complement.*

E. E. Ecker, L. Pillemer and A. O. Kuehn.

From the Institute of Pathology and the University Hospitals of Western Reserve University, Cleveland, Ohio.

Complement is generally inactivated at 56° C for a period of 30 minutes although a much higher temperature is required to destroy its thermostabile components.

During the course of some experiments it was found that NaCl protected the third component and mid-piece (euglobulin) against the action of alkalis, zymin, etc. This observation led to the study of the thermostability of the component parts of complement with and without the addition of NaCl.

Methods. All titrations were done by the method of initial hemolysis described by Ecker, Pillemer, Wertheimer and Gradis.[1]

The inactivation of complement by the action of yeast was effected by a zymin preparation as described by Whitehead, Gordon and Wormall,[2] and of the fourth factor by the method of Gordon, Whitehead and Wormall.[3]

The general procedure for each experiment was as follows: One cc each of fresh guinea pig serum and of the same serum containing 10% NaCl by weight was pipetted into serological (10x1 cm) tubes and heated at temperatures ranging from 54° C to 66° C for a period of 30 minutes. After heating, the salt-containing serums were diluted 10 times with distilled water, and the salt-free serums with 0.9% saline. Equal parts of each of the 1:10 serums were then added to both the zymin-treated and the ammonia-treated serums. The mixtures were allowed to stand at room temperature for 15 minutes, and were titrated by the method of initial hemolysis. The heated serums, by themselves, were completely inactive. The heating was done in an especially constructed water-bath (Dewar flask) with an electrical control unit so that the variations at temperatures in the range of 64° C to 66° C were ±0.1°. At lower temperatures the variations were about ±0.05°.

Summary and Conclusions. From the accompanying graph it is

---

* Aided by a grant from the Commonwealth Fund.

[1] Ecker, E. E., Pillemer, L., Wertheimer, D., and Gradis, H., *J. Immunol.*, 1938, **34**, 19.

[2] Whitehead, H. B., Gordon, J., and Wormall, A., *Biochem. J.*, 1925, **19**, 618.

[3] Gordon, J., Whitehead, H. B., and Wormall, A., *Biochem. J.*, 1926, **20**, 1028.

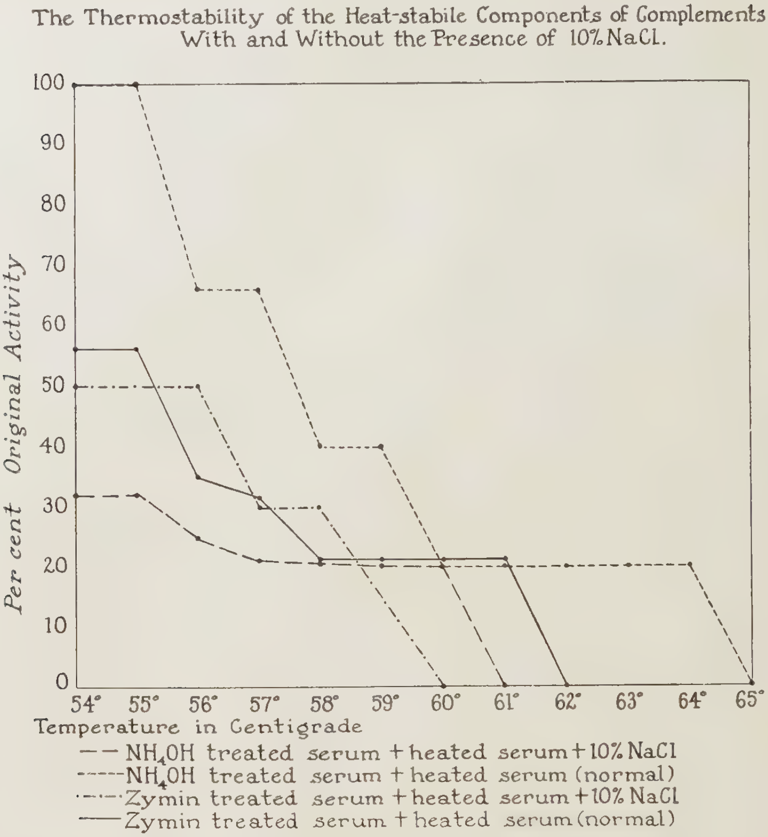


FIG. 1.

seen that the fourth component is relatively more heat-stabile than the third component. The addition of 10% NaCl to the complement prior to heating markedly diminishes the thermostability of the fourth component, while this effect is not noted in the case of the third component, except that in the presence of 10% NaCl the third component is completely inactivated at 60° C while in the absence of the salt a temperature of 62° C is required. It is also observed that the fourth component is inactivated at 65° C while the third component is inactivated at 62° C. From these findings it is evident that the third component is more intimately associated with the serum proteins than is the fourth component. It is therefore suggested that in the routine inactivation of complement for various serological purposes, inactivation be accomplished at 54° C instead of the customary 56° C, provided the temperature control is adequate.

11599

Effects of Body Temperature and of Pentobarbital on Brain Damage Produced by Insulin Shock.*

DAVID B. TYLER. (Introduced by C. H. Thienes)

From the Department of Pharmacology, School of Medicine, University of Southern California.

In most animals there is a fall in body temperature during insulin shock. This fall has been attributed to the lack of available glucose which is essential for the shivering mechanism.[1, 2] In cats, the fall in temperature is very pronounced and, as a rule, drops to about 8° to 10° C above that of the room. It was previously shown[3] that it was necessary to maintain cats in the "medullary phase" (Phase IV) of insulin shock for at least 100 minutes in order to produce irreversible clinical signs of brain damage. In those experiments, the body temperature of the animals ranged from 28° to 33°, representing a drop of 6° to 11° below normal.

During these experiments, it appeared that brain damage occurred earlier in animals resistant to the hypothermic action of insulin. The following experiments were carried out to test this matter further. Some observations on the results obtained from administering sodium pentobarbital (nembutal) during insulin shock are also reported.

Method. Cats fasted 18 hours were given 15 to 20 u/kg of insulin. Fifteen minute records of the neurological state of the animal were made until the animal reached Phase IV, (as described by Frostig.[4] It was then closely watched and when it showed signs of circulatory collapse and extreme respiratory irregularities, small amounts of glucose were given intraperitoneally. This usually restored the animals temporarily to Phase II or III. This glucose administration was repeated again whenever necessary. The body temperature (rectal) in the "warm" cats was maintained at near normal levels either by a hot pad or by working at room temperature of about 30° C. Hypoglycemia was finally terminated with glucose. Those cats which showed irreversible clinical symptoms of cortical or cerebral damage[3, 5] were considered as having brain

---

* We are indebted to Eli Lilly Company for the insulin and to Abbott Laboratories for the sodium pentobarbital (nembutal) used in these experiments.

[1] Dworkin, S., and Finney, W. H., *Am. J. Physiol.*, 1927, **80**, 75.

[2] Tyler, D. B., PROC. SOC. EXP. BIOL. AND MED., 1939, **42**, 278.

[3] Tyler, D. B., and Ziskind, E., PROC. SOC. EXP. BIOL. AND MED., 1940, **44**, 622.

[4] Frostig, J. P., *Arch. Neurol. and Psychiat.*, 1938, **39**, 219.

[5] Ziskind, E., and Tyler, D. B., PROC. SOC. EXP. BIOL. AND MED., 1940, **43**, 734.

damage. Histological data will be presented at a later date.

Results. In the table the results obtained in animals with near normal body temperature were compared with those obtained in animals where the temperature was allowed to fall. The duration of Phase IV is listed in Column I, the temperature range during this period in Column II, the incidence of brain damage in Column III, and the degree of brain damage (+=cortical, ++=cortical plus subcortical, +++=clinical picture of decerebration) in Column IV. It can be seen that when the body temperature was prevented from falling, not only the incidence of brain damage in each group was higher, but the degree of damage produced was greater. In 8 "cool" cats, no damage occurred up to 45 minutes. Of the 5 "warm" cats, 2 terminated after 15 minutes in the medullary phase showed no clinical evidence of brain damage, 3 terminated at 25, 35 and 45 minutes all showed evidence of cortical damage. For periods above 45 minutes, brain damage invariably resulted when the body temperature was kept at normal levels.

In another series of experiments, 10 cats were given sodium pentobarbital (10 mg/kg) when the first myoclonic jerk or convulsion occurred. The administration of this drug abolished the characteristic progression of the symptoms described by Frostig, and the only indications of the cats reaching the medullary phase were by the occurrence of extremely slow pulse and very irregular respiration. Of these animals, 4 died very shortly after the administration of nembutal, confirming the observation of Yannet[6] that animals under this drug are more sensitive to insulin. Six of the cats were observed as described above, glucose being given when the pulse or respiration warranted it. When they had spent a total of 150 minutes in the medullary phase, the hypoglycemia was terminated. All 6 cats, however, remained in a prolonged coma 4 to 6 days in spite of the fact that the hypoglycemia was terminated. Three died

TABLE I.

No. of animals	I Minutes in phase IV	II Temperature range, °C	III Incidence of brain damage	IV Degree of brain damage
8	0-45	31-34	0	0
5	0-45	36-39	60	+
7	46-90	31-34	28	+
5	46-90	36-39	100	++½
8	91-120	31-33	63	++
8	91-120	36-38	100	+++

---

[6] Yannet, H., *Arch. Neurol. and Psychiat.*, 1939, **42**, 395.

on the 4th and 5th days without regaining consciousness. Two of the remaining 3 cats recovered and showed no clinical symptoms of brain damage. The other one recovered with indications of some cortical damage. These preliminary results seem to indicate that sodium pentobarbital, although increasing the sensitivity to insulin, exerts some protection to the brain. In other experiments where sodium pentobarbital was not administered, 150 minutes in the medullary phase usually produced severe cortical and subcortical damage, and the resulting preparations lived about 48 hours.

Discussion and Conclusions. The protective action of cooling during insulin shock has been reported previously.[2] These experiments demonstrate that when the body temperature is prevented from falling, the time necessary to produce damage is considerably shortened, and the degree of brain damage is greater. This is to be expected since the higher the temperature, the higher the metabolic rate and the greater the oxygen need of the brain. When the body temperature is kept at normal levels during insulin shock it would seem that a relatively greater degree of tissue anoxia results than when the body temperature is permitted to fall, due to the greater need of oxygen at higher temperatures. This may be a factor responsible for the prolonged comas and accidents frequently occurring during insulin shock therapy, since the drop in body temperature in humans is not as great as that which occurs in cats. Dogs also are found to be resistant to the hypothermic action of insulin. In this laboratory, it has been found that considerably shorter periods are necessary to produce brain damage in this animal than is needed for cats. In fact, brain damage has been produced in the dog without it showing the characteristic symptoms of the 4th phase.[7]

At this time, no explanation can be offered for the prolonged post hypoglycemic coma which results when sodium pentobarbital is administered to insulinized cats, except perhaps for the known reduction of metabolic rate.[8]

---

[7] Katz, Y., private communication.

[8] Zorn, C. M., Muntwyler, E., and Barlow, O. W., *J. Pharm. and Exp. Therap.*, 1939, **66**, 326.

11600 P

Consistency in Lengths of Post-Ejaculatory Quiescent Periods in Adult Male Rats.*

C. P. Stone, L. W. Ferguson, and C. Wright.

From the Department of Psychology, Stanford University.

In copulating male rats, intromissions usually follow each other in close succession until a vaginal plug is ejaculated.[1] Thereafter, for a brief interval the male is quiet. During the first hour and a half of copulation the quiescent periods typically last from 5 to 10 minutes in potent males that have fully recovered from previous sexual activity. In long tests (2 to 3 hours) the periods gradually lengthen.

Differences in the lengths of quiescent periods for different animals with approximately the same number of ejaculations are much greater than those for the same individuals; in short, there is a degree of consistency that gives to each rat a somewhat characteristic temporal profile of copulatory activities and pauses. Table 1 presents coefficients of correlation between post-ejaculatory quiescent periods or pauses for 57 male albino rats tested in the Stanford laboratory.[2] The males were between 9 and 11 months of age. Thirty-three of them had a 3-hour test and 24, a 2-hour test. Some, but not all, had reached copulatory satiation at the end of their tests; hence some arbitrariness had to be exercised in determining the number of post-ejaculatory pauses to be studied. For this pre-

TABLE I.

Correlations Between Lengths of Successive Post-ejaculatory Quiescent Periods in Male Albino Rats. Below each coefficient of correlation, in parentheses, is given the standard error of that coefficient.

Post-ejaculatory pause	Post-ejaculatory pause				Number of rats
	1	2	3	4	
2	.69 (.10)				57
3	.68 (.10)	.83 (.08)			55
4	.44 (.14)	.63 (.12)	.74 (.10)		45
5	.32 (.21)	.75 (.15)	.70 (.16)	.65 (.17)	23

---

* This study was financially supported by funds from the National Research Council, Committee for Research on Sex Problems.

[1] Lataste, F., *J. de l'Anat. et Physiol.*, 1883, **19**, 144.

[2] Stone, C. P., and Ferguson, L. W., *J. Comp. Physiol.*, 1940, **30**, No. 2.

liminary report we have dealt with only the first 5 pauses, because the number of animals qualifying for consideration of the sixth pause was only 9, a number certainly not representative of the original group.

Correlations for adjacent ejaculatory pauses, for the most part, are somewhat higher than those that are one or more pauses remote, which is what one usually finds in psychobiological functions. For adjacent periods the obtained correlations are similar in magnitude to correlations reported for frequency of copulation by adult male rats in successive tests of from 15 to 30 minutes duration.[3, 4] Also, they compare favorably with measures of consistency of learning scores by rats in maze[5] and serial light discrimination problems,[6] wherein the results from one part of the trial series are compared with those from parts which follow. The authors know of no correlations between any measures of a highly complex psychobiological function in rats that exceed those given in Table 1. Thus, it appears that we have in this post-ejaculatory pause a psychobiological "indicator" which may have a wide range of usefulness in fundamental studies of individual differences. Extensions of the present study along the following lines seem warranted: (1) methodological research to increase the consistency of post-ejaculatory pauses beyond that herein reported, as still higher consistency is desirable for studies of individual differences; (2) investigations of the predictive value of these pauses for other less readily obtained measures of sexual potency; and (3) correlational studies of lengths of post-ejaculatory pauses and other variables, such as chronological age, previous sexual experience, spontaneous motor activity, perseverance and work output, strength of drives in other spheres (*e.g.,* hunger, thirst, sociability), and amounts of male hormone in the blood stream. Space does not permit our elaborating on the significance of such studies, but we hope to deal with some of them in forthcoming papers.

Summary. Correlations between lengths of post-ejaculatory periods of inactivity in adult male rats were found to range between .65 and .83 for adjacent pauses. These correlations are remarkably high for complex psychobiological functions and are not exceeded by those for any other complex functions similarly studied. It seems probable that these post-ejaculatory pauses may be used as a basis for many fundamental studies of individual differences in male rats.

---

[3] Stone, C. P., Barker, R. G., and Tomilin, M. I., *J. Genet. Psychol.,* 1935, **47**, 33.

[4] Anderson, E. E., *J. Comp. Psychol.,* 1936, **21**, 447.

[5] Stone, C. P., and Nyswander, D. B., *Ped. Sem.,* 1927, **34**, 447.

[6] Stone, C. P., *Ped. Sem.,* 1928, **35**, 557.

11601

Delayed Blood Coagulation and Absence of Clot Retraction in Collodion Lined Vessels.

John S. Hirschboeck. (Introduced by P. F. Swindle.)

From the Department of Physiology, Marquette University School of Medicine, Milwaukee, Wis.

The inhibiting influence of certain surfaces upon the blood coagulation process has been known since 1886 when Freund demonstrated an increased coagulation time for blood collected in vaseline lined vessels.[1] Bordet and Gengou[1] in 1903 reported a similar prolongation in paraffin coated tubes. Neubauer and Lampert[2] in 1930 determined the coagulation time for blood in contact with a variety of surfaces. They concluded in harmony with the traditional explanation that, excluding other factors, the coagulation time is inversely proportional to the capacity of the surface to become wet, (Benetzbarkeit) that is, to the adhesive force exerted between the blood and the surface. With glass, where there is much adhesion, the coagulation time is shorter than with paraffin where there is very little adhesion.

The anticoagulant property of a collodion surface has apparently not been previously described.

If a test tube lined with collodion is used as a receptacle for freshly drawn blood, the coagulation is delayed usually as long or longer than when a paraffin lined tube is used. Table 1 presents a comparison of coagulation times in glass, paraffin and collodion lined tubes. The blood was removed with a No. 20 guage needle and a dry syringe from the arm veins of healthy adults. Two cc of blood were placed in each tube, and the coagulation time was taken when the blood failed to flow upon tilting the tube to the horizontal position. In the 12 specimens examined, the coagulation time was definitely longer in the collodion than in the glass tubes. In all but 2 instances it was longer in the collodion than in the paraffin tubes, the average being 4.7 minutes longer for the collodion series.

If the traditional explanation, as formulated by Bordet and Gengou and reaffirmed by Lampert, is true one would expect collodion to have a lower coefficient of adhesion than paraffin for blood. Lampert[3] has indicated that the adhesive forces acting when blood

---

[1] Bordet, J., and Gengou, O., *Ann. Inst. Pasteur*, 1903, **17**, 822.

[2] Neubauer, O., and Lampert, H., *Muench. Med. Wchnschr.*, 1930, **77-1**, 582.

[3] Lampert, H., *Die physikalische Seite des Blutgerinnungsproblems*, 1931, Leipzig, Georg Thieme.

TABLE I.
Coagulation Time of Blood in Lined Test Tubes.

Case	Type of surface		
	Glass	Paraffin	Collodion
1	5 min	11 min	12 min
2	7	26	29
3	4	20	31
4	5	18	25
5	10	23	28
6	8	23	16
7	6	10	15
8	7	13	27
9	7	22	28
9a*	8	19	27
10	5	13	15
10a*	4	13	13

*Same subject repeated two days later.

or distilled water wet a particular surface are relatively the same for each of the two liquids. After using a photographic method for recording the menisci, he calculated the adhesion between distilled water and a variety of materials. He expressed his results in units of "Benetzbarkeit." He listed smooth glass as possessing 42 units, platinum 10 units, athrombit 2.4 units and paraffin —7 units. A minus quantity indicates a negative meniscus. Lampert measured a celloidin surface as possessing 42 units. He did not determine the coagulation time with celloidin or collodion which is essentially the same substance, namely pyroxylin. It is easy to determine the force of adhesion between water and collodion or paraffin by the capillary tube method when Westergren blood sedimentation tubes lined with these materials are used. With the formula $T=hdr/2$, the force of adhesion (T) is expressed in grams of force per centimeter, when h is the height of the liquid in cm, d is the density of the liquid, and r is the radius of the tube By this method the glass-water adhesion is 0.053 g per cm, the collodion-water adhesion is 0.034 g per cm, and the paraffin-water adhesion is —0.037 g per cm. It is evident from this that collodion is an exception to the rule. Its coefficient of adhesion is much higher than that of paraffin and yet blood coagulation is delayed longer with it than with paraffin.

The tubes were allowed to stand for 24 hours at room temperature. There was a complete absence of clot retraction in all of the collodion tubes whereas some retraction took place in all but one of the paraffin tubes. The adherence of the clot to the collodion was so firm that vigorous shaking failed to release it in almost all of the tubes. A little serum collected on the free surface of the clot but never along the tube wall.

Preparation of a Collodion Lined Tube—A test tube 1.5 cm in diameter is filled half full of collodion U.S.P. and then emptied slowly while being rotated. The tube is placed inverted in a wire rack and is allowed to stand for at least 18 hours to give time for the ether and alcohol to completely evaporate. The membrane usually adheres firmly to the glass unless separation begins to take place at the rim.

Summary and Conclusions. The coagulation time of normal human blood on a collodion surface is longer than the coagulation time on a paraffin surface even though the force of adhesion between blood and collodion is much greater than between blood and paraffin. There is no clot retraction in collodion lined tubes.

11602

Attempt to Propagate Poliomyelitis Virus Through Serial Passage in Cotton Rats by "Spreading Factor of Duran-Reynals."

WILLIAM McDOWELL HAMMON. (Introduced by W. L. Aycock.)

From the Department of Preventive Medicine and Epidemiology, Harvard University Medical School and School of Public Health, Boston, Mass.

Numerous attempts have been made to "adapt" poliomyelitis virus to passage in the eastern cotton rat since Armstrong[1] reported that a strain of virus isolated from one of a number of cases occurring during an outbreak of poliomyelitis in Lansing, Michigan, could be propagated in the variety *Sigmodon hispidus hispidus.* These attempts, including those of Armstrong, to obtain takes with other strains of poliomyelitis virus have failed. Toomey[2] has recently reported unsuccessful attempts to propagate 9 other monkey adapted strains of this virus. He inoculated 9 groups of *Sigmodon hispidus littoralis* rats, each with one of 9 strains of virus and later when symptoms failed to develop, these animals were shown to be susceptible to the Lansing strain.

Duran-Reynals[3, 4] has shown that testicular extract ("spreading factor") added to the virus of vaccinia increased the extent of the lesions in the rabbit, and Hoffman and Duran-Reynals[5] showed that it had similar enhancing effects on the viruses of herpes, vesicular stomatitis and Borna disease. In this laboratory several experiments have been made recently to further investigate the effects of testicular extract on the pathogenesis of a number of filterable viruses. Our unreported and incomplete experiments, although of a slightly different character, tend to confirm those reported for the virus of herpes and also suggest that a somewhat similar effect is obtained when the spreading factor is combined with minimal doses of the virus of lymphocytic choriomeningitis given to mice intracerebrally.

---

[1] Armstrong, C., *Pub. Health Rep.*, 1939, **54**, 1719.

[2] Toomey, J. A., and Takacs, W. S., PROC. SOC. EXP. BIOL. AND MED., 1940, **43**, 536.

[3] Duran-Reynals, F., *Compt. rend. Soc. de biol.*, 1928, **99**, 6.

[4] Duran-Reynals, F., *J. Exp. Med.*, 1929, **50**, 327.

It was decided to attempt to adapt a strain of poliomyelitis virus to the cotton rat by suspending the virus in testicular extract, in the hope that this factor might enable the virus to propagate in a tissue that presented conditions which possibly were partially though not entirely suitable for its growth. Since others had failed to adapt any but the Lansing strain, by ordinary methods, our only interest was to test the effect of the "spreading factor" in addition to the standard methods. The possibility that the testicular extract might have an inhibitory effect held no significance for the cotton rat itself was to all practical purposes refractory to the virus.

A portion of cord from a monkey infected with the Toomey "T" strain of virus which was obtained from Dr. McKhann of The Children's Hospital in Boston was ground in a sterile mortar with alundum to make a 10% suspension in a fresh 15% saline extract of rabbit's testicle. After centrifugation at low speed the supernatant was inoculated in doses of 0.06 cc intracerebrally, 0.06 cc intranasally, and 1.00 cc intraperitoneally in each of 3 cotton rats *(Sigmodon hispidus littoralis)*. These animals were obtained from central Florida. Two others were inoculated in a similar manner with testicular extract alone. From the tenth to the twelfth day one of the rats inoculated with virus refused food and appeared irritable. It was killed with ether and the brain removed. One half of this (the inoculated hemisphere) was frozen at —76° C and the other half was ground in a mortar with alundum in 0.5 cc of testicular extract and 1.5 cc of infusion broth, centrifuged slowly and inoculated in the dosage and manner previously described to one other cotton rat. Following this, 4 other such serial passages were made, each at an interval of from 12 to 14 days. In each instance after the tenth or twelfth day it was noted, by recording the weight of the daily food consumption, that the intake had decreased 25% to 50% from the normal for that animal. On the last passage the cotton rat was weighed daily and a slight weight loss was noted after the tenth day. No paralysis was observed in any of these rats nor after the first passage was any symptom noted suggestive of central nervous system involvement.

---

[5] Hoffman, D. C., and Duran-Reynals, F., *J. Exp. Med.*, 1931, **53**, 43.

One half of the brain from the fifth passage (the inoculated hemisphere) was suspended by grinding as previously in 2.0 cc of infusion broth (without spreading factor) and the supernatant after centrifuging inoculated intracerebrally in 0.5 cc doses to two macacus rhesus monkeys. In a similar manner 2 other monkeys were inoculated with a suspension of the frozen brain from the first passage rat. None of the monkeys developed paralysis or any other signs suggesting poliomyelitis. One of those inoculated with the fifth passage brain died on the 22nd day with severe diarrhea associated with an extensive adhesive peritonitis due to infection with *Strongyloides oesophagostomum.* Fifteen days following the inoculation of the monkeys with the first passage brain and 30 days after inoculating the surviving one with the fifth passage brain, all were inoculated intracerebrally on the contralateral side with a 5% suspension in infusion broth of the same monkey cord used to inoculate the original cotton rats. These monkeys all developed typical poliomyelitis, with the onset of symptoms first noted on the sixth and seventh days and progressing to extensive, complete paralysis.

Discussion. It appears that the cotton rat *Sigmodon hispidus littoralis,* when inoculated under the conditions of this experiment with the Toomey "T" strain of poliomyelitis virus, together with the "spreading factor of Duran-Reynals" does not serve as a host capable of permitting the preservation or propagation of the virus. The infectivity of the original material and the susceptibility of the monkeys used to test for the presence of the virus in the brains of the first and fifth passage rats, were controlled by subsequent inoculation with the same virus. The uniformity of loss of appetite in the cotton rats on the tenth or twelfth day after inoculation was the only symptom suggesting any reaction to this strain of virus. It might be considered that the passage interval was too short or too long (it was shorter than the incubation period noted by Armstrong during the early passages of the Lansing strain and longer than that of the fully adapted virus), but if the virus had retained its activity in the brain of the first passage rat its presence should have been detectable at 12 days by monkey inoculation, since the inoculated portion of the cotton rat brain was used to prepare the suspension.

11603 P

Resting Peripheral Blood Flow in Hypertensive Subjects.*

David I. Abramson.

From the May Institute for Medical Research of the Jewish Hospital, Cincinnati, O.

In the course of plethysmographic studies on the peripheral vascular responses of hypertensive patients, it was noted that in about 50% of the cases the resting blood flow in the forearm was much greater than that in subjects with normal blood pressure. Such findings differ from those presented previously by Prinzmetal and Wilson[1] using a similar method, and from those obtained by Pickering,[2] who utilized Stewart's calorimetric procedure.[3] These investigators found that the average blood flow reading for the forearm in a series of hypertensive subjects was no greater than that in a normal group, and on the basis of these observations, together with some confirmatory studies, they concluded that the increased vascular resistance in hypertension is generalized throughout the systemic circulation rather than confined to the splanchnic area. Pickering[4] subsequently pointed out, as did also Stead and Kunkel,[5] that in the light of Grant and Pearson's work,[6] the data included in the two investigations represented not only arterial inflow to the forearm, but also venous return from the hand. It is well known that blood flow through the hand can be affected by a variety of stimuli,[7] and hence readings obtained under such conditions cannot be considered representative of peripheral blood flow generally. Further, as Prinzmetal and Wilson indicated, the opposite conclusion to the one presented by them, namely that the hypertonus is limited to the splanchnic region, could have been drawn if an increase in blood flow had been found in the forearm of hypertensive subjects of the magnitude of 1.5 times the normal figure, *i.e.*, approximately 2.65 cc per min. per 100 cc of limb volume instead of 1.7 cc.[1]

---

* Aided by the Samuel and Regina Kuhn Fund, and a grant from the Dazian Foundation for Medical Research.

[1] Prinzmetal, M., and Wilson, C., *J. Clin. Invest.*, 1936, **15**, 63.

[2] Pickering, G. W., *Clin. Sc.*, 1936, **2**, 209.

[3] Stewart, G. N., *Heart*, 1911, **3**, 33.

[4] Pickering, G. W., *Proc. Staff Meet. Mayo Clinic*, 1939, **14**, 310.

[5] Stead, E. J., Jr., and Kunkel, P., *J. Clin. Invest.*, 1940, **19**, 25.

[6] Grant, R. T., and Pearson, R. S. B., *Clin. Sc.*, 1938, **3**, 119.

[7] Capps, R. B., *J. Clin. Invest.*, 1936, **15**, 229; Kunkel, P., Stead, E. A., Jr., and Weiss, S., *J. Clin. Invest.*, 1939, **18**, 225; Abramson, D. I., and Ferris, E. B., Jr., *Am. Heart J.*, 1940, **19**, 541.

In the experiments herein reported, care was taken to maintain a pressure of at least 350 mm Hg at the wrist during blood flow determininations in the forearm, so that venous return from the hand could not affect the results.[6] The bath temperature was kept at 32° C and the room temperature at 25° C; the technic used being similar in all respects to that previously reported.[8] The results ob-

TABLE I.
Resting Blood Flow in the Forearm.
Hypertensive Subjects.

Case No.	Age years	Blood flow	Blood pressure mm Hg	Case No.	Age years	Blood flow	Blood pressure mm Hg
1	64	2.1	$\frac{172}{92}-\frac{218}{90}$	15	74	3.4	$\frac{164}{86}-\frac{192}{92}$
2	31	2.0	$\frac{170}{110}-\frac{200}{110}$	16	43	4.0	$\frac{160}{100}-\frac{158}{98}$
3	71	3.3	$\frac{160}{80}-\frac{205}{102}$	17	29	1.9	$\frac{156}{108}-\frac{190}{130}$
4	43	4.6	$\frac{218}{120}-\frac{236}{134}$	18	70	3.3	$\frac{182}{90}-\frac{202}{110}$
5	72	5.9	$\frac{160}{90}-\frac{230}{112}$	19	40	1.7	$\frac{160}{100}-\frac{192}{110}$
6	52	6.3	$\frac{236}{104}-\frac{264}{110}$	20	57	3.0	$\frac{230}{108}-\frac{270}{140}$
7	45	4.0	$\frac{150}{88}-\frac{180}{90}$	21	67	6.1	$\frac{226}{126}-\frac{236}{132}$
8	62	2.1	$\frac{166}{100}-\frac{170}{120}$	22	64	4.0	$\frac{230}{110}-\frac{230}{130}$
9	54	1.6	$\frac{160}{100}$	23	38	4.0	$\frac{230}{140}-\frac{240}{140}$
10	46	1.2	$\frac{162}{130}$	24	51	1.4	$\frac{220}{122}-\frac{250}{118}$
11	64	1.4	$\frac{158}{80}-\frac{170}{100}$	25	23	2.5	$\frac{160}{94}$
12	53	2.6	$\frac{230}{140}$	26	59	1.4	$\frac{180}{110}-\frac{192}{122}$
13	44	4.7	$\frac{152}{102}-\frac{208}{110}$	27	31	1.5	$\frac{180}{124}$
14	69	3.1	$\frac{220}{120}-\frac{230}{130}$	28	41	3.0	$\frac{204}{124}$

Avg—3.1 cc

---

[8] Abramson, D. I., Zazeela, H., and Marrus, J., *Am. Heart J.*, 1939, **17**, 194, 206; Ferris, E. B., Jr., and Abramson, D. I., *Am. Heart J.*, 1940, **19**, 233.

Case No.	Age years	Blood flow	Blood pressure mm Hg	Case No.	Age years	Blood flow	Blood pressure mm Hg
Subjects with Normal Blood Pressure.							
1	52	1.8		20	25	3.3	
2	27	1.9		21	26	2.5	
3	20	2.0		22	37	1.2	
4	18	1.3		23	33	1.8	
5	27	1.5		24	36	1.5	
6	34	1.5		25	25	0.9	
7	26	1.8		26	20	1.8	
8	18	2.2		27	30	1.8	
9	36	2.2		28	19	2.7	
10	28	1.5		29	25	2.2	
11	42	0.9		30	23	1.5	
12	43	1.9		31	15	1.2	
13	50	1.1		32	19	1.7	
14	56	1.1		33	23	1.6	
15	30	2.2		34	36	1.7	
16	19	0.8		35	28	1.0	
17	24	1.7		36	53	2.3	
18	30	3.1		37	74	1.6	
19	26	2.5		38	77	2.2	

Avg—1.8 cc

Bath temperature—32°C. Blood flow in cc per min per 100 cc limb vol.

tained in a series of 28 hypertensive patients of various types were compared with those in a series of 38 normal subjects. The average forearam blood flow in the latter (Table I) was found to be 1.8 cc per min. per 100 cc of limb volume, while that for the hypertensive group (Table 1) was 3.1 cc (1.7 times greater than that for the control series). In the case of 4 known hypertensive subjects, but in whom normal blood pressure readings were obtained in the period in which the tests were performed, the average forearm blood flow was 1.8 cc.

In view of the disparity between our results and those reported by other investigators, it becomes necessary to reëxamine their conclusion that an increased vascular tonus exists at the periphery in hypertension.

11604

Effect of Lipid Solvents on Fourth Component of Complement.*

L. Pillemer, S. Seifter and E. E. Ecker

From the Institute of Pathology and the University Hospitals of Western Reserve University, Cleveland, Ohio.

Toda and Mitsuse[1] and Tokano[2] have influenced several investigators[3] to associate the fourth component of complement with a lipid fraction of serum. This is at such variance with the concept that the fourth component is the carbohydrate-complex carried by the end-piece,[4] that it was decided to repeat in detail the experiments of Toda and Mitsuse.

Methods and Materials. The methods advocated by Toda and Mitsuse for the inactivation of the fourth component by ether, chloroform, benzene, benzine and cadmium chloride were followed in detail, and without the slightest variation. All reagents were Merck Blue Label chemicals. Methods for complement titration and reactivations have already been given.[4]

Results. Under the exact conditions employed by Toda and Mitsuse, ether and chloroform destroyed complement irreversibly, but had no specific effect on the fourth component. Benzene and benzine did not inactivate dehydrated complement. Furthermore, the treatment of serum with cadmium chloride (1% in 0.9% NaCl) caused flocculation of the serum proteins, and no reactivation of either the supernatant liquid or the redissolved precipitate was possible. Varying the concentration of either the cadmium chloride or the serum also failed to reveal any specific effect on the fourth component.

It becomes evident that these findings do not confirm the work of Toda and Mitsuse, and that the fourth component is neither a lipid nor associated with lipids. Previous work from this laboratory[5] had also established that prolonged extraction of dehydrated, active complement with fat solvents does not inactivate complement; instead, an improvement of the lytic titer is obtained when 50% of

---

* Aided by a grant from the Commonwealth Fund.

[1] Toda, T., and Mitsuse, B., *Z. f. Immunitätsforsch.*, 1933, **78**, 62.

[2] Tokano, Y., *Z. f. Immunitätsforsch.*, 1936, **87**, 29, 72.

[3] Osborne, T. W. B., *Complement or Alexin*, London, Oxford University Press, 1937.

[4] Pillemer, L., Seifter, J., and Ecker, E. E., in press.

[5] Ecker, E. E., Pillemer, L., and Grabill, F. G., Proc. Soc. Exp. Biol. and Med., 1938, **38**, 318.

the phospholipids is removed. Treatment of fresh serum under the same conditions results in an irreversible inactivation of the total complement due to protein denaturation.

While it is agreed that the phospholipids of the mid-piece may play a role in complementary activity,[6] it has not been possible to establish a lipid-fourth component relationship.

Summary. The fourth component of complement was not found to be in association with a lipid complex of serum.

11605

Effects of Irradiation on Leukemic Cells in Marrow Cultures.*†

Edwin E. Osgood. (With the technical assistance of Evelyn Packham.)

From the Department of Medicine and the Division of Experimental Medicine, University of Oregon Medical School, Portland.

The experiments herein reported were undertaken to determine whether a small dose of irradiation would have a selectively greater effect than a large dose on a susceptible cell, such as the lymphocyte, in comparison with a less susceptible cell, such as the progranulocyte[1] (promyelocyte); whether an increase in dose of irradiation produced a corresponding increase in effect; and whether leukemic cells were more susceptible than nonleukemic cells of the same type.

The marrow culture technic[2] permits quantitative studies of the effect of irradiation on living normal or leukemic human cells. After an initial total and differential cell count, 50 cc of culture containing about 100,000,000 nucleated cells in a medium consisting of 65% balanced salt solution and 35% human cord serum is thoroughly mixed, and equal portions of about 8 cc are placed in each of several 30 cc vaccine vials. One of these is left as a control, and the others

---

[6] Ecker, E. E., Jones, C. B., and Kuehn, A. O., in press.

* Aided by a grant from the Dazian Foundation. Dr. S. E. Rees and Dr. W. Haworth kindly irradiated some of these cultures.

† Presented before Section N of the American Association for the Advancement of Science, Seattle, Washington, June 19, 1940.

[1] The cell nomenclature used is the same as that which is found in Osgood, E. E., and Ashworth, Clarice M., *Atlas of Hematology*, pp. 6-8, J. W. Stacey, Inc., San Francisco, 1937; and Osgood, E. E., *A Textbook of Laboratory Diagnosis*, pp. 161-164, Ed. 3, The Blakiston Company, Philadelphia, 1940.

[2] Osgood, E. E., and Brownlee, Inez E., *J. A. M. A.*, 1937, **108**, 1793.

are given various doses of irradiation. All vials are handled identically except for the irradiation. The total and differential cell counts are repeated at intervals. Curves are plotted expressing the absolute number of each type of cell in the irradiated cultures in percentage of the number of the same type of cell in the control at the same time.

In a previous publication[3] it was shown that irradiation, in the doses used therapeutically, apparently does not kill living human

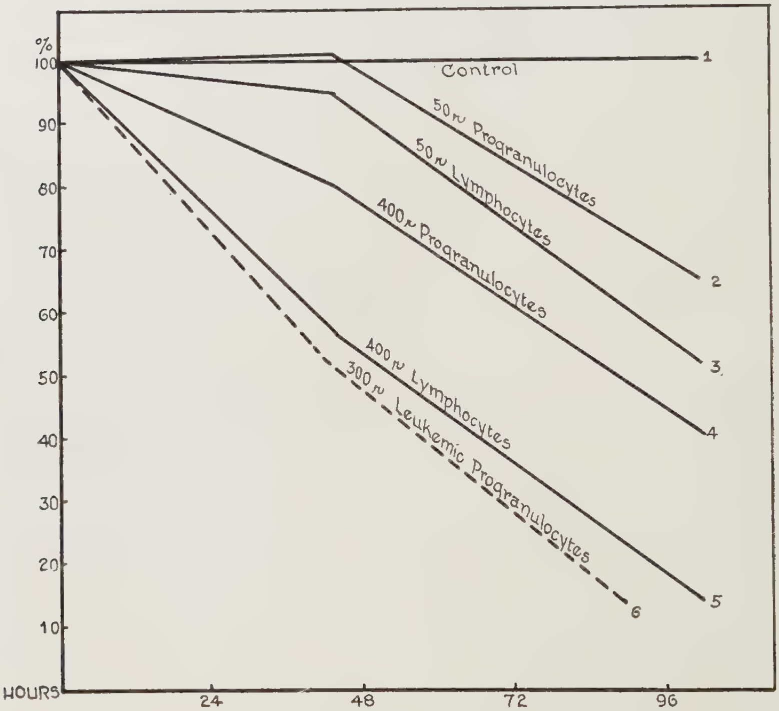


FIG. 1.

Effects of Irradiation on Progranulocytes and Lymphocytes. Curve 1 corresponds to the controls for all experiments, since the other curves are plotted in percentage of the absolute number of the same type of cells in the control at the same time. Curves 2-5 are the weighted averages for the cultures from eight experiments in which in addition to control vials an identical vial was given 50 r of irradiation and another vial 400 r of irradiation. The cell type and dosage of irradiation are indicated for each curve. Curve 6 is from a culture of the blood of a patient with subacute granulocytic (myelogenous) leukemia which contained 93% of progranulocytes A, in which one vial served as a control and an identical vial received 300 r of irradiation. In all experiments irradiation was given at 200 KV, 7 MA, 1 mm copper, and 1 mm aluminum, irradiation at 40 cm distance, 22 r per minute, and 0.063 A minimum wave length, and a half value layer of 1.54 mm copper. The dosage recorded is that measured with a thimble ionization chamber inside a vial from which the bottom had been cut off.

---

[3] Osgood, E. E., and Bracher, G. J., *Ann. Int. Med.*, 1939, **13**, 563.

cells in marrow cultures, but does prevent the onset of mitotic or amitotic division. This results in a straight line decrease in progranulocytes and lymphocytes which continues for a period of several days. The more mature cells of the granulocyte series begin to decrease only after sufficient time has elapsed for maturation of progranulocytes to this stage. This suggests that irradiation does not directly affect the more mature cells which are not capable of division.

In Fig. 1, curves 2-5 were obtained by adding the absolute numbers of progranulocytes and lymphocytes in the controls and irradiated vials from 8 experiments, and then computing several points on each curve in percentage of the absolute number of cells in the control at the same time intervals. In each of the 8 experiments there was one control vial, one vial which was given 50 r of irradiation, and one vial which was given 400 r of irradiation, with the factors indicated in the legend. Therefore, any deviation from the 100% control line represents a difference due to the irradiation.

Curve 6 was obtained from a culture of the blood of a patient with subacute granulocytic leukemia which contained 93% of progranulocytes A^1 (promyelocytes II). All of these cells were peroxidase positive and had too coarse a chromatin structure to be blast cells, and yet contained either no granules or azurophil granules alone with no neutrophil, eosinophil or basophil granules. Because of the high percentage of progranulocytes the probable error of any point on the curve is relatively small.

Note from curves 2-5, Fig. 1, that lymphocytes from the same cultures were more affected by the same dose of irradiation than were progranulocytes; that all of the curves approach a straight line decrease beginning at the time of irradiation; and that the dose of 50 r of irradiation had a lesser effect than the dose of 400 r, but not in direct proportion to the dose; *e.g.:* from curve 2 at 102 hours there are 35% less progranulocytes in the cultures irradiated with 50 r than in the control, while from curve 4 there are 60% less progranulocytes in the cultures irradiated at 400 r than in the control, which is a ratio of 35 to 60 or 1 to 1.7. Similarly, from curves 3 and 5 the ratio of percentage decrease in lymphocytes is 48 to 85 or 1 to 1.8, but the ratio of 50 r to 400 r is 1 to 8, so that it is obvious that increasing the dose of irradiation does not produce a directly proportional increase in effect.

It seemed possible since lymphocytes are more affected than progranulocytes that a more selective action on the most susceptible cell would be obtained by decreasing irradiation. That this is not the case is shown by the fact that the ratio of decrease in progranulocytes to decrease in lymphocytes with 50 r of irradiation is 35 to 48 or 1 to 1.4 which is, within the limits of error of the method, the same as the ratio of decrease in progranulocytes to decrease in lymphocytes with 400 r of irradiation which is 60 to 85 or 1 to 1.4.

From curve 6 in Fig. 1 it is apparent that the general type of curve obtained with leukemic progranulocytes is similar to that obtained with nonleukemic progranulocytes, which suggests that the mechanism of action is the same,[3] that is by arrest of mitotic division, permitting these cells to live out their normal life span and then to die. However, the slope of the curve is steeper than the slope of curve 4 which averages 8 experiments on nonleukemic progranulocytes, and is slightly steeper than the steepest of the curves from any of the 8 individual experiments. This suggests, but does not prove, that the effect of irradiation is quantitatively greater on leukemic cells than on nonleukemic cells. The most plausible explanation for this would be a naturally greater rate of cell division and a shorter life span for the leukemic cells.

It was interesting to note that neither in the irradiated nor in the control cultures of this leukemic blood, even though normal cord serum was used in the medium, was there any tendency for the progranulocytes to mature (Table 1), although maturation has occurred in this type of medium with all of the more than 500 cultures we have made of nonleukemic cells or cells from chronic leukemias in which the mature stages are found in the blood. This suggests that the failure of maturation of the cells in acute and subacute leukemias is not due to any alteration in their environment but is due to a fundamental change in the cell itself, and supports the view that these are malignant cells.[4]

TABLE I.
Percentage of All Cells of the Granulocyte Series More Mature Than the Progranulocyte in the Experiment from Which Curve 6, Fig. 1, Was Obtained.

Time in hr after irradiation	0	18	66	90
Control	1.2	1.1	0.9	1.6
300 r	1.2	0.8	2.3	4.0

The failure of these cells to mature with irradiation is strong evidence against Isaacs' theory[5] that irradiation acts by hastening maturation to a stage at which cell division is no longer possible. However, our studies of the effects of irradiation on normal cells and cells from chronic leukemias explain Isaacs' observation that after irradiation the proportion of mature cells in the cell population is increased, for as our experiments with colchicine showed[3] X-rays prevent the onset of mitotic division. The immature cells capable of such division are therefore bound to decrease in numbers first, and only later the more mature cells developed from them.

---

[4] Furth, J., *J. Exp. Med.*, 1935, **61**, 423; Osgood, E. E., and Ashworth, Clarice M., *Atlas of Hematology*, p. 36, J. W. Stacey, Inc., San Francisco, 1937.

[5] Isaacs, R., *Arch. Int. Med.*, 1932, **50**, 836.

Summary. In irradiated marrow cultures of nonleukemic cells the ratio of effect of 50 r of irradiation on progranulocytes to the effect on the more susceptible lymphocyte is the same as with a dose of 400 r of irradiation. An increase of 8 times in the amount of irradiation, that is from 50 r to 400 r, produced an increase of only about 1.7 times in the effect on either progranulocytes or lymphocytes. Leukemic progranulocytes showed a greater decrease from a dose of 300 r than nonleukemic progranulocytes from a dose of 400 r, but the character of the curves is similar which suggests that the mechanism of action of irradiation on leukemic cells is probably a prevention of the onset of cell division as was shown to be the case for nonleukemic cells. Leukemic progranulocytes which failed to mature in the patient also failed to mature in cultures in a medium containing normal human cord serum with or without irradiation. All of these observations are explained if the action of irradiation is to prevent the onset of mitotic or amitotic division and not by directly killing cells and if the leukemic process depends on a fundamental change in the cell affecting its rate of division and maturation and not on any alteration in the environment.

11606

Microscopic Lesions Without Functional Impairment of Striated Musculature of Suckling E-Low Rats.*

Ira R. Telford, Gladys A. Emerson and Herbert M. Evans
From the Institute of Experimental Biology, University of California, Berkeley.

In endeavoring to determine the minimal prophylactic dose of alpha tocopherol which would insure the normality of the cross-striated musculature in suckling young rats, alpha tocopherol dis-

---

* Aided by grants from the Research Board and the Department of Agriculture of the University of California, the Rockefeller Foundation, and Merck and Company, Rahway, New Jersey. Assistance was rendered by the Works Progress Administration, Official Project No. 65-1-08-62, Unit A-5.

solved in ethyl laurate was administered at the 6 mg and 10 mg levels by stomach tube to a considerable series of mothers on the first day of lactation. The young were observed daily for evidences of the slightest impairment of gait, capacity to right themselves promptly when placed on their backs, et cetera. Thirty young were thus studied from mothers receiving 6 mg of alpha tocopherol and 41 from mothers receiving 10 mg. Sixty-four young from mothers receiving the solvent alone were also observed; in the last mentioned group all young showed paralyses or evident impairment of muscular function. Virtually all the young from mothers receiving either dose of alpha tocopherol appeared to be perfectly normal in their gait, righting reactions, et cetera.

Histological study of 16 muscles was made in the case of several animals from each of the 3 groups. To our surprise in the case of the 6-mg group, 12 of the 16 muscles examined showed the typical microscopic lesions of dystrophy, although these were not extensive; *i.e.* plenty of normal musculature remained, a fact in harmony with the complete lack of observable motor impairment of these animals.† In the 10-mg group microscopic lesions were entirely absent from all muscles studied. The muscles examined were diaphragm, gastrocnemius, adductor magnus, semimembranosus, extensor digitorum longus (communis), flexor digitorum profundus, biceps brachii, triceps brachii, psoas major, erector spinae group, sternomastoideus, serratus anterior, acromiotrapezius, pectoralis major, masseter and tongue. The masseter and tongue musculature proved themselves to be the least likely to be affected, the gastrocnemius and sternomastoideus the most likely to be affected.

---

† After this manuscript was sent for publication a paper by A. M. Pappenheimer (*Journal of Mt. Sinai Hospital,* 1940, **7**, 65) appeared which carries the statement that histological degeneration of the musculature was observed in young rats exhibiting none of the usual symptoms of E avitaminosis.

11607

Studies of Drugs in Tissue Metabolism. I. Method for Measuring Metabolism of Tissue in Serum.

Dale G. Friend and A. Baird Hastings

From the Department of Biological Chemistry, Harvard Medical School, Boston.

The action of compounds of pharmacological importance on the oxidative systems of tissue cells has received widespread attention, particularly through the work of Jowett and Quastel[1] and of Krahl and Clowes.[2] The usual procedure is to determine the metabolism of excised tissue suspended in a balanced salt solution. However, since it might be expected that the respiration of tissue would proceed more normally if the tissue were suspended in a naturally occurring physiological fluid, our attention was first directed to the development of a simple, reproducible method of measuring tissue respiration in serum. A description of this method provides the content of the present paper. Rat liver was chosen as the standard tissue whose respiration was measured, and human blood serum as the nutrient fluid medium in which the tissue was suspended.

Experimental. Indirect methods for determining the respiration of tissue in serum are already available.[3, 4] However, they have the disadvantage of being complicated and requiring more than the usual equipment for the determination of O_2 consumption by the direct Warburg technic.

The direct measurement of oxygen consumption of tissue in serum has been achieved by (a) alternately acidifying and evacuating serum until its CO_2 content has been reduced to about 0.5 mM per liter (2% of original value); (b) readjusting its pH to about 7.2-7.3; (c) using this CO_2-free serum as the fluid medium in which the tissue is immersed; (d) measuring the O_2 consumption of the tissue manometrically in the Erlenmeyer type Warburg chamber, with absorption of CO_2 by alkali. In this method, the pH of the fluid medium does not change outside the physiological limits, due to the buffering action exerted by the plasma proteins. Use of serum in the direct Warburg method without removal of CO_2 and subsequent adjustment of pH is not feasible due to the high pH which develops as the CO_2 is absorbed by the alkali.

---

[1] Jowett, M., and Quastel, J. H., *Biochem. J.*, 1937, **31**, 565.

[2] Krahl, M. E., and Clowes, G. H. E., Jr., *J. Cell. Comp. Physiol.*, 1938, **11**, 21.

[3] Dickens, F., and Simer, F., *Abderhalden's Handb.*, 1933, **4**, 435.

[4] Dixon, M., *Biochem. J.*, 1937, **31**, 924.

Details of Technic. Approximately 25 cc samples of blood were drawn from fasting normal human subjects, under sterile conditions, and transferred to a tube containing approximately 5 cc of mineral oil. Both the tube and the mineral oil were previously sterilized by autoclaving for 15 minutes under 20 lb pressure. The blood was centrifuged and the serum transferred to a sterile 1000 cc separatory funnel containing mineral oil. (The presence of the oil was found to be necessary to eliminate frothing during evacuation.)

It was found by repeated trials that the addition of 0.25 cc of 0.1 N HCl per cc of serum, followed by evacuation for 30 minutes at a pressure of 4-10 mm Hg, reduced the CO_2 content of the serum to about 0.5 mM per liter, with a final pH of approximately 7.25. Determinations with the glass electrode showed that the serum pH was between 4 and 5 immediately after addition of the acid. The subsequent evacuation removed the CO_2 and restored the pH to normal.

Serum treated in this way is ready for use as a nutrient medium. Dixon[4] recommended the removal of species antibodies by heating to 56°C for 2½ hours. Human serum can be heated in this manner without obtaining the jelling which occurs in certain species. However, since it was found, in a series of 24 observations with heated and unheated human serum, that there was a negligible difference in the O_2 consumption of rat liver, the results to be reported in the present studies were obtained on unheated serum.

Slices of rat liver were prepared according to Warburg's procedure[5] and placed immediately in CO_2-free, phosphate Ringer's solution prepared according to Dickens and Simer.[3] Slices approximately 0.3 mm thick, totalling from 70 to 100 mg in weight, were then transferred to the Erlenmeyer type Warburg vessels containing 2.6 cc of the CO_2-free serum, and 0.4 cc of 20% KOH in the central cup. The vessels were attached to the manometers, and filled with oxygen. All the above procedures were carried out in a constant temperature room maintained at 38°.

The vessels were shaken in a water bath maintained at 38°, and after temperature equilibrium had been established, the manometers were read at 10-minute intervals for one hour. The tissue was then removed, dried at 110° for 24 hours and the dry weight determined. The oxygen consumption, Q_{O_2}, was calculated in terms of cubic millimeters per milligram of dry weight per hour. It was found that the rate of respiration of the liver in serum was uniform up to 100 minutes, which is longer than one finds for liver in Ringer's solution.

---

[5] Warburg, O., *Biochem. Z.*, 1924, **152**, 51.

TABLE I.
Oxygen Consumption of Rat Liver in Human Serum.

Rat No.	Initial serum CO_2 content mM/L	Initial serum pH	Final serum pH	Q_{O_2} cmm/mg/hr
1	.40	7.26	7.56	—12.5
		7.26	7.46	—12.7
2	.50	7.25	7.38	—11.7
		7.25	7.36	—11.4
3	.53	7.28	7.54	—11.8
		7.28	7.52	—11.9
		7.28	7.56	—11.8
		7.28	7.73	—12.5
		7.28	7.72	—12.6
		7.28	7.72	—12.1
4	.52	7.10	7.57	—11.3
		7.10	7.60	—11.7
5	.50	7.12	7.67	—14.7
		7.12	7.69	—14.5
6	.24	7.18	7.32	—12.5
		7.18	7.34	—12.2
		7.18	7.34	—12.4
7	—	7.28	7.43	—12.6
		7.28	7.45	—13.0
		7.28	7.42	—12.7
		7.28	7.43	—12.7
Mean	.48	7.23	7.51	—12.5 ± 0.6

Results. Determination of liver metabolism. Table I shows the results obtained in 21 determinations on the livers of 7 different rats. The mean value of Q_{O_2} was 12.5 ± 0.6; minimum 11.3, maximum 14.7. This is 25% higher than the value of 10 usually found in our laboratory for the Q_{O_2} of normal rat liver in Ringer's solution. The average difference in the results obtained on any one rat was only about 3%. The pH of the serum measured with the glass electrode was from 0.2 to 0.5 higher at the end of the experiment than at the beginning, but was still within the normal pH range. This increase in pH occurred whether or not tissue was present during the incubation.

It was concluded from this series of experiments that the present method of studying liver metabolism in serum was suitable for studying the effect of adding different materials to the serum. It also seemed to be a suitable method for determining whether serum as drawn contains toxic materials.

11608

Studies of Drugs in Tissue Metabolism. II. Action of Drugs on Metabolism of Tissue in Serum.

DALE G. FRIEND. (Introduced by A. Baird Hastings.)

From the Department of Biological Chemistry, Harvard Medical School, Boston.

The present paper is a study of the action of certain drugs on surviving tissue cells, respiring in serum. Specifically, it deals with the effect of the hypnotics, chloral hydrate and barbital; and of certain dyes, particularly methylene blue, on the respiration of liver tissue in human serum. The experimental technic employed in this study has been described in the preceding paper. All experiments were performed in duplicate or triplicate.

Methylene Blue. Barron has shown that this dye stimulates the oxygen consumption of red blood cells and of cells poisoned with KCN,[1, 2] but does not increase the oxygen consumption of normal liver or kidney tissue suspended in phosphate-Ringer solution.[2] The effect of this dye on the oxygen consumption of liver when added to serum was, therefore studied in order to determine whether liver in serum might not carry out oxidative reactions not possible in a simple salt solution. Using human serum, we found, to our surprise, that there was an increase of approximately 50% in oxygen uptake upon adding the dye in a concentration of 0.5 mg/cc, while dye concentrations of 0.05 mg/cc and 5.0 mg/cc gave an increase of approximately 20%, as shown in Table I.

On the other hand, in confirmation of Barron,[2] we found that methylene blue added to phosphate-Ringer's caused no increase in the oxygen consumption of rat liver. Nor did the addition of 4 other dyes (Congo red, rose Bengal-blue, brilliant vital red and Evans blue, all of which are sometimes used in clinical work) affect the

TABLE I.
Effect of Adding Methylene Blue to Serum on Oxygen Consumption of Rat Liver.

Rat No.	Conc. dye mg/cc	Avg Q_{O_2} without dye	Avg Q_{O_2} with dye	% increase
1	.05	—11.5	—14.1	22.6
2	.5	—12.3	—18.3	48.8
3	.5	—11.0	—16.9	53.6
4	.5	—12.4	—17.5	41.0
5	.5	—11.4	—16.9	48.2
6	.5	—14.6	—21.8	49.3
7	5.0	—12.1	—14.3	18.2

---

[1] Barron, E. S. G., and Harrop, G. A., Jr., *J. Biol. Chem.*, 1928, **79**, 65.

[2] Barron, E. S. G., *J. Exp. Med.*, 1930, **52**, 447.

metabolism of liver suspended in serum. It is concluded from these observations that the reversibly oxidizable dye, methylene blue has the ability in the presence of serum of increasing the oxygen consumed by rat liver slices *in vitro*. What portion of the oxidation system is affected is not known. It is interesting to note that in serum a large dose of the dye (5 mg/cc) does not give as much oxygen consumption as the smaller dose of 0.5 mg/cc, thus indicating that such large doses may be toxic to the tissue.

Chloral Hydrate. Chloral hydrate has been shown by Jowett[3] to exert a depressing effect on the oxygen consumption of brain cells, in doses ranging from 0.15-0.9 mg/cc using phosphate-Ringer's media. We have studied the effect of toxic concentrations of chloral hydrate in serum, (3.3 mg/cc) on the oxygen consumption of liver. As shown in Table II, there was a marked decrease, approximately 68%, in the oxygen consumption of liver tissue which is in agreement with comparable observations made by Jowett and Quastel,[4] on the action of such hypnotics.

Chloral Hydrate and Methylene Blue. In view of the observation by Barron[2] that certain reversible dyes will increase the metabolism of cells after inhibition by certain poisons, and of our observation that methylene blue stimulates oxygen consumption of liver tissue in serum media, this dye was added to serum containing chloral hydrate to determine whether it exerted any effect on the oxygen consumption of tissue after its depression by the narcotic. These results are shown in Table III.

TABLE II.
Action of Chloral Hydrate (3.3 mg per cc) on Rat Liver Suspended in Human Serum.

Rat No.	Q_{O_2} without chloral	Q_{O_2} with chloral	% depression of Q_{O_2}
1	—12.5	—4.3	65
2	—12.5	—4.1	67
3	—13.5	—4.6	66
4	—14.6	—3.4	77

TABLE III.
Action of Methylene Blue in Concentration of 0.5 mg per cc on Rat Liver Poisoned with Chloral Hydrate.

Rat No.	Conc. of chloral hydrate mg/cc	Q_{O_2} without dye	Q_{O_2} with dye	% increase in Q_{O_2}
1	2.0	—3.7	— 5.3	43.3
2	3.3	—4.6	— 8.4	82.6
3	3.3	—6.0	—14.0	133.0
4	3.3	—4.3	— 9.3	116.0

[3] Jowett, M., *J. Physiol.*, 1938, **92**, 322.

[4] Jowett, M., and Quastel, J. H., *Biochem. J.*, 1937, **31**, 565.

It was found, as this table shows, that the dye greatly increased the oxygen consumption after inhibition by chloral hydrate, in some experiments restoring the Q_{O_2} to an approximately normal value. Whether or not this means that the normal oxidation processes of the cells were restored cannot be stated, although it is probable that the chloral inhibited a necessary, reversibly oxidizable component which was replaced by the dye.

Sodium Barbital (sodium di-ethyl-barbiturate) and Methylene Blue. In view of the observations of Jowett and Quastel[4] that luminal (phenobarbital) and evipan (sodium N-methylcyclohexenylmethylbarbiturate) depress the oxygen consumption of brain and liver in phosphate-Ringer's, a study was made of the action of one drug of this series (sodium barbital) in serum media. The results are presented in Table IV. It was found that barbital in a concentration of 3.3 mg per cc depressed the metabolism of liver in serum from 13 to 34%. In contradistinction to the stimulating effect of methylene blue on the metabolism of liver after chloral hydrate, the dye had no such effect on the barbital depressed tissue. Indeed, there was a further decrease in oxygen consumption. This is to be interpreted as indicating that the barbital decreased the oxidative processes of the tissue cells by inhibiting quite a different portion of the oxidation system than that affected by the chloral.

Comment. The observations reported above indicate that the method of measuring the metabolism of rat liver in human serum is suitable for the study of the action of drugs on the oxidative system of tissue cells. It has also been shown by the opposite effects produced by a reversibly oxidizable dye, such as methylene blue, on the metabolism of liver depressed by chloral and by barbital, that a difference in their mode of action is indicated. It is, perhaps, of interest that in 3 comparable experiments with morphine added to serum in rather large amounts (1 mg per cc), no effect on the respiration of liver was found. So far, no study has been made of serum from patients who had received the drugs studied in the present paper.

TABLE IV.
Action of Sodium Barbital (Concentration 3.3 mg per cc) and Methylene Blue (Concentration .5 mg per cc) on Oxygen Consumption of Liver in Serum.

Exp. No.	Q_{O_2} with barbital cmm/mg/hr	% depression*	Q_{O_2} with barbital and dye cmm/mg/hr	% depression*
1	10.9	13	10.2	18
2	9.9	21	7.0	44
3	8.2	34	6.4	49

*Standard normal liver $Q_{O_2} = 12.5$.

However, it has been shown by Friend and Robinson,[5] using this technic, that sodium thiocyanate exerts a depressant action on liver metabolism both when added to serum *in vitro* and when present in blood serum as drawn. A hitherto unpublished experiment, furthermore, showed that serum from a patient to whom a large amount of thiocyanate had been administered depressed the liver metabolism 30%. The addition of methylene blue to this serum restored the liver metabolism to its normal Q_{O_2} of 13.0.

Summary. The effect on liver metabolism of adding methylene blue, chloral hydrate and sodium barbital to serum has been studied. Methylene blue has been found to increase the oxygen consumption of rat liver respiring in human serum. Chloral hydrate and sodium barbital both depress the metabolism. Following depression of the oxygen consumption by chloral hydrate, methylene blue increases the metabolism. Following sodium barbital, the dye further depresses the metabolism. This is interpreted as indicating a difference in the site of action of the drugs on the oxidative system of the tissue cells.

11609

Excretion and Determination of Cinchophen in Bile.

W. B. BRADLEY AND A. C. IVY.

From the Department of Physiology and Pharmacology, Northwestern University Medical School, Chicago.

It has been established that cinchophen has a choleretic action in man and dog.[1-7] It does not apparently stimulate bile volume output in the rabbit.[8] The effect of cinchophen on the output of the various constituents of bile is disputed in the literature,[1-7] and good quantitative data on the excretion of cinchophen in the bile could not be found by us. However, cinchophen is known to be excreted in bile;[2]

---

[5] Friend, D. G., and Robinson, R. W., *J. Lab. Clin. Med.*, 1939, **24**, 832.

[1] Brugsch and Horsters, *Z. ges. exp. Med.*, 1923, **38**, 367.

[2] Taubmann, *Arch. exp. Path. u. Pharm.*, 1927, **121**, 204.

[3] Chabrol and Maximin, *Press. Med.*, 1929, **37**, 666.

[4] Taschenberg and Hofmann, *Deut. med. Wochn.*, 1925, **51**, 1611.

[5] Speerling and Hartman, *J. Lab. and Clin. Med.*, 1928, **13**, 854.

[6] Horsters, *Arch. exp. Path. u. Pharm.*, 1925, **105**, xi.

[7] Goffin, *Compt. rend. Soc. de Biol.*, 1936, **123**, 97.

[8] Strausky, *Biochem. Z.*, 1926, **155**, 256.

in fact, diiodoatophen (2-o-iodophenyl-6 iodoquinoline-4 carboxylic acid, or biloptin) has been used for cholecystography.[9, 10]

Our work was primarily undertaken to determine the rate and extent of the excretion of cinchophen in bile. At the same time, however, the choleretic action and effect of cinchophen on the output of various bile constituents was compared with sodium dehydrocholate.

Methods. The effect of cinchophen on bile flow and the excretion of cinchophen in the bile was studied by using 5 anesthetized dogs and 4 "chronic" bile fistula dogs prepared by the Rous-McMaster method. In the "acute" experiments the dogs were anesthetized with pentobarbital. The common bile duct was cannulated and the cystic duct tied. Bile was collected for periods of one hour each. After the secretion had become constant, cinchophen (as the sodium salt) was injected intravenously in doses of 50 mg/K of body weight. The volume of bile and the amount of cinchophen in the bile were determined for hourly intervals for a period of 5 hours after the injection of cinchophen. Five other anesthetized dogs were also used to study the effect of sodium dehydrocholate.

Bile was collected from the "chronic" bile fistula dogs for 24-hour periods. After the volume output of bile had become constant, one gram of cinchophen was fed daily for 15 days. The volume of bile and the amount of cinchophen in the bile were determined for 24-hour periods.

The method devised for the determination of cinchophen in bile follows: A 1 ml sample of bile is pipetted into a 6-inch test tube. Eight drops of concentrated NaOH are added together with a few drops of caprylic alcohol to prevent foaming and a few beads to prevent bumping. The mixture is refluxed for 2 hours after which 1 ml of saturated $NaNO_2$ is added through the condenser followed by 3 ml of concentrated HCl. The mixture is again refluxed for 15 minutes and then evaporated down to half its volume in an oil bath at 115°C. The contents of the tube are then cooled and filtered quantitatively with suction through filter paper pulp into a 15 ml conical centrifuge tube, the test tube and filter being washed repeatedly with small quantities of 1% HNO_3. At this point the volume of the filtrate is about 13 ml. One ml of 10% phosphomolybdic acid is added to precipitate the cinchophen. If there is no precipitate there is no cinchophen in the sample.

---

[9] Pribram, *Deut. med. Wochn.*, 1926, **52**, 1291.

[10] Einhorn and Stewart, *Med. J. and Rec.*, 1926, **125**, 457; *Ibid.*, 1927, **126**, 430.

If there is a precipitate, it is centrifuged down and washed twice by centrifugalization with a few ml of 1% HNO_3. The washed precipitate is dissolved in 5 ml of N/10 NaOH and is transferred quantitatively into a 100 ml volumetric flask. Enough wash water should be used to make the volume about 60 ml. Twenty ml of 20% potassium ferrocyanide and 10 ml of concentrated HCl are then added and the contents of the flask made up to volume with distilled water.

A standard is prepared by pipetting into a 15 ml conical centrifuge tube 1 or 2 ml of a standard solution of recrystallized cinchophen (as the sodium salt) per ml. Ten ml of 1% HNO_3 are added followed by 1 ml of 10% phosphomolybdic acid. The resulting precipitate is treated in the same manner as the unknown.

After standing for 30 minutes the unknown is matched against the standard in a colorimeter. The quantity of cinchophen in the unknown is calculated by dividing the reading of the standard by the reading of the unknown and multiplying by the number of mg of cinchophen in the standard.

Normal bile yielded no cinchophen by this method. Cinchophen added to normal bile could be recovered quantitatively with an error of not more than 5%. The cinchophen used in this work was a white, odorless powder, soluble in alkaline solution (Calco Chemical Co.).

TABLE I.
Effect of Cinchophen and Dehydrocholic Acid on Bile Formation.
The data represents the averaged results obtained on 5 dogs.

		Bile pigment		Cholic acid		Cholesterol		Cinchophen	
Hr	Vol. cc	Mg/ 100 cc	Total Mg	Mg/ 100 cc	Total Mg	Mg/ 100 cc	Total Mg	Mg/ 100 cc	Total Mg
Control	1.98	263.0	4.66	2120.0	37.2	19.9	.314		
50 g/kilo cinchophen intravenously. Avg 5 dogs.									
1st	12.4	109.7	14.0	264.0	32.6	4.66	.589	169	22.3
2nd	11.7	70.7	8.21	85.5	9.0	3.80	.430	237	28.6
3rd	10.2	70.7	7.16	89.0	7.9	3.64	.356	195	20.0
4th	9.02	78.0	6.95	79.7	6.6	3.81	.329	164	15.0
5th	7.9	94.5	6.61	132.0	9.03	8.64	.578	146	11.9
									97.8 mg total
Avg wt—10.1 K.									
Control	2.19	489.0	11.6	2230.0	33.2	17.2	.261		
50 mg/kilo sodium dehydrocholate intravenously. Avg 5 dogs.									
1st	16.4	130.0	22.1	451.0	56.2	4.29	.532		
2nd	5.31	129.0	7.19	563.0	24.6	6.41	.289		
3rd	3.03	202.0	7.24	755.0	17.7	9.07	.264		
Avg wt—10.7 K.									

The bile was analyzed for total pigment by the method of Schmidt and Jones,[11] for cholic acid by the method of Reinhold and Wilson,[12] and for cholesterol by the method of Bloor.[13]

Results. Anesthetized dogs. The averaged results of the "acute" experiments are shown in Table I. It is to be noted that by weight cinchophen given intravenously is a better choleretic than sodium dehydrocholate (Decholin). It is well known that sodium dehydrocholate is superior in regard to choleretic action than sodium cholate and the conjugated cholates. During the first hour after the injection of cinchophen, the total output of pigment, cholic acid and cholesterol was definitely increased. The same occurred after sodium dehydrocholate. During the remainder of the period of observation, the chief difference between the 2 choleretics pertained to volume output and cholic acid output. Cinchophen caused a more prolonged choleretic action and depressed cholic acid output.

Cinchophen was excreted in relatively large amounts. The average dose given was 505 mg, and the average amount recovered during the 5-hour period following its injection was 97.8 mg, or almost 20% The maximum excretion occurred during the second hour, but significant amounts were still being excreted during the fifth hour.

"Chronic" biliary fistula dogs. In these experiments we were particularly interested in obtaining data regarding the amount of cinchophen excreted daily in the bile when 100 mg per kilo body weight of cinchophen were given orally with the meal. This is definitely a toxic dose of the drug. Such a dose is used to produce experimental gastric or duodenal ulcer and causes gastritis with anorrhexia and frequently vomiting after 2 to 4 days of administration. For that reason, valid data on the effect of cinchophen in the doses used, on cholic acid, pigment and cholesterol output could not be obtained.[14] Regardless of anorrhexia and the consequent failure to consume all the food, the animals showed a choleresis of from 50 to 150% above the control level.

Reliable data for the output of cinchophen in the bile could be obtained usually during the first day or two after the initiation of the medication, following which reliable data could be obtained only for the days during which vomiting was known not to occur. The average daily recovery of cinchophen in the bile of the different dogs was: dog 1, 28%; dog 2, 78%; dog 3, 74%; and dog 4, 42%. These averages represent the data for the days during which vomiting did not occur. The average of all the results yields a recovery of 55%. Twenty-four hours after cessation of the administration of the drug, it could not be detected in the bile.

---

[11] Schmidt, Jones and Ivy, PROC. SOC. EXP. BIOL. AND MED., 1936, **34**, 17.

[12] Reinhold and Wilson, *J. Biol. Chem.*, 1932, **96**, 637.

[13] Bloor, *J. Biol. Chem.*, 1928, **77**, 53.

[14] Schmidt, Beazell, Berman and Ivy, *Am. J. Physiol.*, 1939, **126**, 120.

Discussion. We were primarily interested in ascertaining the extent to which cinchophen is excreted in the bile when given in doses sufficient to cause gastritis and gastro-duodenal ulcer. It is clear from the results that cinchophen is excreted in the bile soon after it enters the blood stream. About 55% of the drug, when given orally, is eliminated daily in the bile. In the biliary fistula dog, cinchophen is excreted in the bile 24 hours after the last dose. This indicates that a considerable quantity of cinchophen may normally undergo an enterohepatic circulation.

Our results on the cholic acid output after the intravenous injection of cinchophen, show that it produces a choleresis without an increase in cholic acid synthesis. In fact, cholic acid synthesis is definitely depressed by the doses used. The same result obtained in the "chronic" bile fistula animals. In 3 of the 4 dogs, the output of cholic acid was definitely decreased. These data are not tabulated because they are equivocal. They are equivocal because the decrease in cholic acid occurred with an anorrhexia or refusal or vomiting of food. These factors are well known to decrease cholic acid output.[14] However, in some instances the cholic acid output was decreased to 0.3 g per day, and in our experience the normal fasting bile fistula dog never excretes less than 0.8 g per day. Yet, cinchophen in the doses used causes a gastritis, and gastritis *per se* may reduce cholic acid synthesis. Although we believe, on the basis of the results of the "acute" experiments, that cinchophen in the doses used directly depresses cholic acid synthesis, we doubt that our evidence on the "chronic" biliary fistula dogs warrants a definite conclusion to that effect. If a dose of cinchophen can be found which will depress cholic acid synthesis without altering the intake and digestion of food, then it will, we believe, be possible to conclude that cinchophen directly interferes with cholic acid synthesis.

Summary and Conclusions. A method for the quantitative determination of cinchophen in bile is described. In 5 anesthetized dogs weighing about 10 kilos, it was found that 20% of 1 g of intravenously injected cinchophen is excreted in the bile in 5 hours. In 4 "chronic" bile fistula dogs an average of 55%, or from 28 to 78%, of orally administered cinchophen (100 mg per kilo body weight) was daily excreted in the bile. This shows that the liver is significantly concerned in the excretion of cinchophen and that an enterohepatic circulation of cinchophen may occur. By weight sodium cinchophen increases the volume output of bile more than sodium dehydrocholate, which is an excellent hydrocholeretic. Cinchophen in large doses orally or intravenously decreases cholic acid output. But, it cannot be concluded from our results on "chronic" bile fistula dogs that cinchophen specifically interferes with cholic acid synthesis.

11610

Roentgen-Pigmentation in the Gold Fish.*

FRIEDRICH ELLINGER.

From the Radiotherapy Department and the Neuropathological Division, Montefiore Hospital for Chronic Diseases, New York City.

One of the outstanding biologic effects of roentgen rays is the pigmentation of the skin. Roentgen pigmentation has been observed not only in human beings but in many mammals and in fish. Most characteristic is its persistence for weeks, months and years. The investigations of Miescher[1] and Peck[2] revealed the importance of chromatophores in this phenomenon. With respect to the rôle which chorial chromatophores play in the roentgen pigmentation of mammals, the eruption of chorial melanophores in the goldfish *(Carassius auratus)* after exposure to roentgen rays as described by Smith,[3, 4] is of interest.

Smith used a radiation of 100 kV, 5 MA, no filter and exposed in some instances only a portion, in other instances, the entire goldfish. Depending upon the dose, after a latent period of 5-6 days eruption of melanophores on the exposed side was observed, a general cutaneous melanosis was brought about in some cases, and some fish finally died. The eruption of melanophores usually however is a transient affair according to Smith. By a process of degeneration these melanophores disappear after 2-4 weeks and the cutaneous regions once more assume a normal color.

---

* Aided by a grant from the Committee in Aid of Displaced Foreign Physicians.

[1] Miescher, G., *Arch. f. Derm.*, 1922, **39**, 313.

[2] Peck, S. M., *Arch. Derm.*, 1930, **21**, 916.

[3] Smith, G. M., *Am. J. Cancer*, 1932, **16**, 863.

[4] Smith, G. M., *Biol. Bull.*, 1932, **43**, 484.

During studies concerning the suitability of goldfish for problems of experimental radiation therapy[5] some observations concerning the roentgen pigmentation have been made. The technic has been described previously.[5] In addition a radiation of 200 KV, 25 MA, 0.5 mm Cu and 3.0 mm A1 Filter, HVL 0.9 mm Cu has been used. The results, based on the observation of more than 2000 goldfish, may be summarized as follows:

(1) The minimum dose to produce pigmentation with roentgen rays of HVL 0.233 mm to 1.0 mm Cu has been found at 800 r (air) under our experimental conditions. (2) In many instances considerably higher doses failed to produce pigmentation. This is in agreement with the results of Smith. (3) The pigmentation started on the head, spread to both sides of the trunk and was pronounced at the lateral lines, in spite of the fact that irradiation always took place in the dorso-ventral position. (4) Pigmentation was more marked when high doses were applied, where the fish's dorsal fin showed some black pigment prior to irradiation, and during spring. (5) Black pigmentation after irradiation extended into the "silver spots" which prior to treatment lacked the red pigment. It has also been observed in the albinotic variety of Carassius, the so-called silver-fish. Twenty-one days after exposure to roentgen rays, the fish appeared light grey. Pigmentation in the albinotic variety of the goldfish produced by mechanical means has been described by Osterhage.[6] These observations show that in the albinotic goldfish, radiation and trauma act in the same manner, as described by Smith[3, 4] for the red variety.

The observation of roentgen pigmentation in albinotic goldfish is especially interesting because of the mechanism of this process: In mammals this phenomenon is explained on the assumption of a photochemical process. In fish this explanation does not seem to hold true. Contrary to albinotic mammals, albinotic fish develop roentgen pigmentation. This directs attention to the fact that the chromatophores of fish are controlled by the nervous system. It is generally known that chromatophores of fish have a double innervation through sympathetic fibres (chromatodilatatores and chromatoconstrictores), and that there exists a chromatomotor nerve center in the medulla oblongata.[7]

Some brains of irradiated goldfish were studied in coöperation with Dr. Charles Davison of the Neuropathological Division. Depending on the dose, there were observed in the region of the medulla oblongata a more or less definite area of gliosis as evidenced by formation of glia fibers. A fuller account of the neurologic changes after roentgen irradiation in the goldfish brain will be given elsewhere.

---

[5] Ellinger, F., PROC. SOC. EXP. BIOL. AND MED., 1939, **41**, 527.

[6] Osterhage, K. H., *Z. mikr. anat. Forsch.*, 1932, **30**, 551.

[7] Literature, see Parker, G. H., *Cold Spring Harbor Symposia on Quantitative Biology*, 1936, Vol. 4.

The destruction of the medullary region by roentgen rays may explain the roentgen pigmentation of the goldfish:

Schaefer[8] was able to demonstrate in Pleuronectes that only cuts into or below the medulla oblongata effected darkening of the caudal segments (expansion of chromatophores because of lack of chromatoconstrictor impulses). The conclusion may, therefore, be drawn that the destruction of the medulla oblongata by roentgen rays acts in the same manner.

If a central disturbance is assumed in the pigment-motor nervous system, this would explain: (1) the start of pigmentation on the head of the fish; (2) the spread of pigmentation on the trunk especially there, where the lateral sense organ is located, whose relation to the nervous-system is known (Smith,[9] Sand[10]); (3) the pigmentation outside of the direction of the incident beam; (4) the pigmentation of albinos; (5) the latent period, slowly developing changes are most characteristic for the effects of roentgen rays on the central nervous system; (6) finally this would explain why in most of our experiments no restoration of the normal color within 28 days has been observed. An injury of the chromato-motor nerve center in the brain can most likely not be repaired; this regeneration or repair may occur, if only the peripheral part has been injured.

Summary. Roentgen pigmentation in mammals and fish have to be considered as different phenomena. While the former is influenced directly by a photochemical process and therefore generally limited to the site of action of roentgen rays, the latter seems to depend on a disturbance of the chromato-motoric nervous system and, therefore, may extend over the total innervated area.

---

[8] Schaefer, J. G., *Arch. f. ges. Physiol.*, 1921, **188**, 25.

[9] Smith, G. M., *Biol. Bull.*, 1930, **58**, 313.

[10] Sand, A., *Proc. Roy. Soc. B*, 1937, **123**, 472.

11611

Phospholipid Partition (Lecithin, Cephalin and Sphingomyelin) of Blood in Pernicious Anemia and Lipemia.

Harold H. Williams, Betty Nims Erickson, Samuel S. Bernstein and Icie G. Macy.

From the Research Laboratory of the Children's Fund of Michigan, Detroit.

The development of micromethods for the determination of the individual phospholipids, lecithin, cephalin and sphingomyelin[1] has made possible investigations of the phospholipid distribution in blood. Characteristic patterns for the phospholipid partition among the 3 separate fractions appear to exist for normal plasma, erythrocytes and tissues,[1] however, preliminary analyses presented in this paper demonstrate that certain anomalies occur in pernicious anemia and lipemia.

A marked reduction of the total phospholipid in plasma is a salient feature in pernicious anemia.[2] That some abnormality in the type of phospholipids likewise occurs has been indicated by lowered recoveries of the phospholipid with petroleum ether, following evaporation of the alcohol-ether extracts in air.[3] In view of data which showed that diminished recoveries accompanied lower fatty acid carbon: phosphorus ratios of the precipitated phospholipids (theoretical ratios for lecithin, cephalin and sphingomyelin being 13.9, 13.9 and 7.4, respectively) a higher proportion of sphingomyelin in pernicious anemia was indicated. Recent data from this laboratory have demonstrated that both lecithin and sphingomyelin may be refractory to petroleum-ether extraction under certain conditions, whereas cephalin is extracted completely.[1]

The data given in the table were obtained on petroleum-ether extracts prepared under carefully controlled conditions of evaporation of the preliminary alcohol-ether extracts which effect a far more complete recovery of the phospholipids[4] and confirm the marked reduction of total plasma phospholipid in pernicious anemia.[2] This decrease is reflected in all three phospholipid components. With treatment and partial relief of the anemia the total phospholipid content of the plasma is raised, accompanied by corresponding increases in lecithin and sphingomyelin, but with a decrease in cephalin content. When complete remission is attained the total phospholipid is above normal; both cephalin and sphingomyelin are likewise elevated to levels far above normal, whereas the absolute amount and proportion of lecithin is conspicuously diminished.

---

[1] Erickson, B. N., Avrin, I., Teague, D. M., and Williams, H. H., *J. Biol. Chem.*, 1940, **135**, 671.

[2] Williams, H. H., Erickson, B. N., Bernstein, S., Hummel, F. C., and Macy, I. G., *J. Biol. Chem.*, 1937, **118**, 599.

[3] Avrin, Ira, Studies on Blood Phospholipids, thesis submitted in partial fulfillment of the requirements for Master of Science in the Department of Chemistry, Wayne University, Detroit, 1937.

[4] Williams, H. H., Erickson, B. N., Avrin, I., Bernstein, S. S., and Macy, I. G., *J. Biol. Chem.*, 1938, **123**, 111.

The total and individual phospholipids of the erythrocytes are slightly reduced in the severe anemia; however, with treatment,

TABLE I.
Phospholipid Partition in Plasma and Erythrocytes of Blood Samples.

	Total phospholipid	Choline phospholipid		Lecithin		Cephalin		Sphingomyelin	
Plasma	mg/ 100 cc	mg/ 100 cc	%*	mg/ 100 cc	%*	mg/ 100 cc	%*	mg/ 100 cc	%*
Normal†	189	134	70	99	52	55	30	35	18
Pernicious anemia									
Severe	47	37	80	33	70	10	21	5	10
	70	36	51	14	21	34	49	21	30
	99	60	61	48	48	39	39	12	12
Partial remission	120	99	82	72	59	22	18	27	23
	124	92	74	61	49	32	26	31	25
Complete ,,	208	102	49	28	13	106	51	74	36
	214	111	52	32	15	103	48	79	37
Lipemia									
Diabetic	741	741	100	668	90	0	0	73	10
,, remission	401	401	100	354	88	0	0	47	12
Essential	1232	961	78	888	72	271	22	73	6
Schüller-Christian	163	126	77	56	34	38	23	70	43
Cells	mg/ 100 g	mg/ 100 g	%	mg/ 100 g	%	mg/ 100 g	%	mg/ 100 g	%
Normal†	317	127	39	77	23	190	61	50	16
Pernicious anemia									
Severe	274	99	36	60	22	175	64	38	14
Partial remission	271	100	37	45	16	170	63	56	21
	180	47	26	8	4	134	74	39	22
Complete ,,	356	86	34	20	6	271	76	65	18
	353	106	30	25	7	247	70	81	23
Lipemia									
Diabetic	307	126	41	99	32	181	59	27	9
,, remission	227	93	41	59	26	134	59	34	15
Essential	337	138	41	—	—	199	59	—	—
Schüller-Christian	321	125	39	38	12	196	61	87	27

*Percent of total phospholipid.
†Average of 4 individuals.[1]

marked alterations in the phospholipid partition, similar to those in the plasma, are produced, *i. e.,* an elevation in total phospholipid, cephalin and sphingomyelin to values higher than normal with the amount of lecithin decidedly decreased. A similar increase of ether-insoluble phospholipid (sphingomyelin) in plasma and cells during treatment of pernicious anemia has been reported by Kirk, although no consistent changes in other phospholipids were observed.[5]

In contrast to the changes in pernicious anemia are those accompanying the elevated total phospholipid in diabetic and essential lipemia.[6] Not only the absolute amount of lecithin but also its proportion in the total was increased. Cephalin was higher, but not proportionately so, in the essential lipemia, whereas no cephalin whatever was found in the plasma of the diabetic lipemia. Sphingomyelin was increased slightly in the plasma of both. The phospholipid partition of the erythrocytes, however, was essentially normal.

Study of an individual with Schüller-Christian disease indicates that in this condition there is a tendency toward an increased amount of sphingomyelin, with a compensatory decrease of lecithin in both plasma and cells.

These preliminary data indicate the need for further investigation concerning the physiological rôles of the individual phospholipids, lecithin, cephalin, and sphingomyelin, in health and disease.

11612

Relation Between Cerebroside and Neutral Fat Contents of Blood Plasma, Erythrocytes and Stroma.

BETTY NIMS ERICKSON, HELEN J. SOUDERS, MARION L. SHEPHERD, D. MAXWELL TEAGUE AND HAROLD H. WILLIAMS.
(Introduced by Icie G. Macy.)

From the Research Laboratory of the Children's Fund of Michigan, Detroit.

That cerebrosides may exist in small amounts in blood has been shown by the development and application of a titrimetric microprocedure for this group of lipids.[1, 2, 3] This method, slightly modified, has been employed on lipid extracts of blood, stroma and brain. The data indicate that in erythrocytes, stroma, and brain, much of the lipid which formerly was designated as neutral fat* is cerebroside, whereas in plasma only negligible amounts of cerebroside are present. Furthermore, there are indications that the cerebrosides may comprise a significant fraction of the blood lipids in certain pathological conditions.

---

[5] Kirk, E., *Am. J. Med. Sci.*, 1938, **196**, 648.

[6] Bernstein, S. S., Williams, H. H., Hummel, F. C., Shepherd, M. L., and Erickson, B. N., *J. Pediat.*, 1939, **14**, 570.

[1] Kimmelstiel, P., *Biochem. Z.*, 1929, **212**, 359.

[2] Kirk, E., *J. Biol. Chem.*, 1938, **123**, 613.

[3] Kirk, E., *J. Biol. Chem.*, 1938, **123**, 637.

Method. Procedure for hydrolysis: The procedure of Kirk[2] was modified to facilitate hydrolysis of the cerebrosides and filtration of the hydrolysate. Aliquot samples of the lipid extract and blanks for initial and total reduction values[2] are measured into 10 cc glass-stoppered graduated cylinders. The solvents are evaporated carefully in a boiling water bath. To each of the samples for initial reduction are added 3 cc of water, and to each of those for total reduction 2 cc of 6 *N* hydrochloric acid. The cylinders are stoppered tightly and heated for 15 minutes in a boiling water bath to emulsify the lipids and hydrolyze the cerebrosides. After cooling, 1 drop of phenol red, 0.5 cc of 4% zinc sulfate solution ($ZnSO_4.7H_2O$), and 2 cc of 0.1 *N* sodium hydroxide are added to each sample for initial reduction, after which they are diluted to 10 cc and filtered through a medium weight filter paper. To each sample hydrolyzed with hydrochloric acid are added 1 drop of phenol red indicator and, in succession, the following: 40% sodium hydroxide (drop by drop) until the solution turns pink; 1 *N* hydrochloric acid until a yellow color is evident; and 0.1 *N* sodium hydroxide until a pink color develops. Finally, 0.5 cc of the 4% zinc sulfate solution and 2 cc of 0.1 *N* sodium hydroxide are added, the samples diluted to 10 cc and filtered through the medium weight paper.

Determination of reduction values: The sugar reduction values are determined with potassium ferricyanide according to the procedure of Hagedorn and Jensen.[4] Aliquots of the filtrates are measured into 1 by 4 inch titer tubes. Exactly 2 cc of 0.02 *N* potassium ferricyanide solution† are added to every sample. The titer tubes are covered with agate balls (1 inch diameter), heated in a boiling water bath for 15 minutes and cooled. Just before titration, 3 cc of iodide-sulfate-chloride reagent,‡ 2 cc of 3% acetic acid and a few drops of starch solution are added. Titration with standard 0.005 *N* sodium thiosulfate solution, to the disappearance of the blue color, is carried out with a microburette. The reduction values in terms of galactose and cerebrosides are calculated according to Kimmelstiel[1] and Kirk.[2]

---

* Heretofore in determinations of the lipid partition of blood and tissues, all of the lipid unaccounted for as phospholipid or cholesterol and its esters has been included in the neutral fat fraction.

[4] Hagedorn, H. C., and Jensen, B. N., *Biochem. Z.*, 1923, **135**, 46; *ibid.*, 1923, **137**, 92.

† Potassium ferricyanide solution—1.65 g of potassium ferricyanide and 10.6 g of fused sodium carbonate in 1000 cc of water.[4]

‡ Iodide-sulfate-chloride solution—10 g of zinc sulfate and 50 g of sodium chloride plus water to make 200 cc. Just before using add 0.5 g of potassium iodide per 20 cc of solution.[4]

Results and Discussion. Cerebroside analyses of plasma and erythrocytes are given in Table I. Small amounts of cerebrosides were detected in normal plasma and in plasma samples from polycythemia, Schüller-Christian disease, and diabetic lipemia; in the latter the neutral fat fraction was elevated ten-fold. A slight elevation of plasma cerebroside accompanied remission of pernicious anemia.

The cerebroside content of normal erythrocytes averaged 47 mg per 100 g of cells, thus comprising 9% of the total lipid (Table I). The red cells in severe pernicious anemia possessed an increased amount of cerebrosides which was further elevated during partial remission and returned to a more normal concentration with a complete remission. Similar observations have been noted by Kirk.[3, 6]

TABLE I.
Cerebroside and Neutral Fat Content of Plasma and Erythrocytes.

		Cerebrosides		Neutral fat*		Neutral fat minus cerebrosides	
	No. cases	mg	%†	mg	%†	mg	%†
Plasma (per 100 cc)							
Normal	4	15	2	225‡	31‡	210	29
’’ §	13	42	8				
Pernicious anemia							
Severe	3	17	4	121	30	104	26
Partial remission	1	31	8	107	28	76	20
Remission	1	47	10	70	15	23	5
Diabetic lipemia	2	27	0.2	1854	39	1827	39
Polycythemia	4	9	1	69	20	60	19
Schüller-Christian	1	2	0.4	148	27	146	27
Erythrocytes (per 100 g)							
Normal	4	47	9	51‡	10‡	4	1
Normal§	13	51	12				
Pernicious anemia							
Severe	1	85	21	123	30	38	9
Partial remission	1	131	25	131	25	0	0
Remission	1	103	16	103	16	0	0
Diabetic lipemia	2	400	50	400	50	0	0
Polycythemia	4	56	8	110	14	54	6
Schüller-Christian	1	69	13	69	13	0	0

*Includes cerebrosides.
†% of total lipid.
‡Values from previous studies.[5]
§Values from Kirk.[3]

---

[5] Williams, H. H., Erickson, B. N., Bernstein, S., Hummel, F. C., and Macy, I. G., *J. Biol. Chem.*, 1937, **118**, 599.

[6] Kirk, E., *Am. J. Med. Sci.*, 1938, **196**, 648.

TABLE II.
Cerebroside and Neutral Fat Content of Stroma and Brain.

		Cerebrosides		Neutral fat*		Neutral fat minus cerebrosides	
	No. cases	mg	%†	mg	%†	mg	%†
Stroma (per 100 mg dry)							
Normal							
Human	5	1.7	14	1.3‡	12‡	0	0
Sheep	1	2.8	11	2.8	11	0	0
Horse	1	1.0	8	1.0	8	0	0
Polycythemia—human	7	1.9	9	3.0	15	1.1	6
Embryo—beef	6	1.6	8	2.1	10	0.5	2
Horse							
Tetanus antitoxin	4	1.4	8	3.2	19	1.8	11
Diphtheria antitoxin	3	1.1	7	1.4	9	0.3	2
Brain (per 100 mg, dry)							
Dog							
Normal§	2	4.0	10	12.0	28	8.0	18
Disulphide anemia§	2	7.9	19	11.6	28	3.7	9

*Includes cerebrosides.
†% of total lipid.
‡Values from previous studies.[7]
§Values from previous studies.[8]

In diabetic lipemia, a large part of the erythrocyte lipid consisted of cerebrosides (50% of the total lipid). This is in striking contrast to observations made on plasma, which contains only a small quantity. The erythrocytes in polycythemia and Schüller-Christian showed no abnormalities in cerebrosides.

Cerebrosides appear to be a structural component of the erythrocyte, for measurable amounts are present in the stroma (Table II). Similar to the lipid distribution of the intact erythrocyte, about 10% of the total stroma lipid—previously classified as neutral fat—now seems to consist entirely of cerebrosides. Likewise, the cerebroside fraction composed 10% of the total lipid of brain tissue, although this value does not account for the total amount (30%) of neutral fat.

---

[7] Erickson, B. N., Williams, H. H., Bernstein, S. S., Avrin, I., Jones, R. L., and Macy, I. G., *J. Biol. Chem.*, 1938, **122**, 515.

[8] Williams, H. H., Erickson, B. N., Beach, E. F., and Macy, I. G., *J. Lab. Clin. Med.*, in press.

11613

Growth-Stimulating Activity of Alpha Tocopherol.*

MARJORIE M. NELSON, GLADYS A. EMERSON AND HERBERT M. EVANS.

From the Institute of Experimental Biology, University of California, Berkeley.

A factor necessary for the normal growth of rats has been reported by several investigators[1-6] to be either vitamin E or a substance closely related to it. Evans, Emerson, and Emerson[7] found that alpha tocopherol stimulated growth in female rats which had plateaued in weight on an E-low diet when it was given at a level of 1 mg daily (6 times weekly) for 50 days. It was felt that the feeding of lower levels of alpha tocopherol might contribute to our understanding of the quantitative relationship between the antisterility and growth-stimulating activity of vitamin E.

A sample of synthetic alpha tocopherol (from Merck and Company) assayed with E-low rats of proven sterility, resulted in 100% littering when given in a single 3 mg dose on the day of positive mating.

Six litters of female rats of the Long-Evans stock were placed on the standard E-low diet 427. After approximately 130 days on the diet, they plateaued at an average weight of 237 g. At 153 days of age they were divided into equivalent groups of 6 littermates each and fed the following supplements daily (6 times weekly) for 135 days:

Group I—80 mg ethyl laurate (Controls).
Group II— 0.25 mg α-tocopherol in 80 mg ethyl laurate.
Group III— 0.50 mg α-tocopherol in 80 mg ethyl laurate.

The growth-stimulating activity of the two comparatively low levels of alpha tocopherol is clearly shown (Fig. 1). The growth

---

* Aided by grants from the Research Board and the Department of Agriculture of the University of California, the Rockefeller Foundation, and Merck and Company, Rahway, N. J. Assistance was rendered by the Works Progress Administration, Official Project No. 65-1-08-62, Unit A-5.

[1] Evans, H. M., *J. Nutr.*, 1928, **1**, 23.

[2] Mason, K. E., *J. Nutr.*, 1929, **1**, 311.

[3] Blumberg, H., *J. Biol. Chem.*, 1935, **108**, 227.

[4] Olcott, H. S., and Mattill, H. A., *J. Biol. Chem.*, 1936, **114**, LXXVII.

[5] Olcott, H. S., and Mattill, H. A., *J. Nutr.*, 1937, **14**, 305.

[6] Emerson, G. A., and Evans, H. M., *J. Nutr.*, 1937, **14**, 169.

[7] Evans, H. M., Emerson, G. A., and Emerson, O. H., PROC. SOC. EXP. BIOL. AND MED., 1938, **38**, 197.

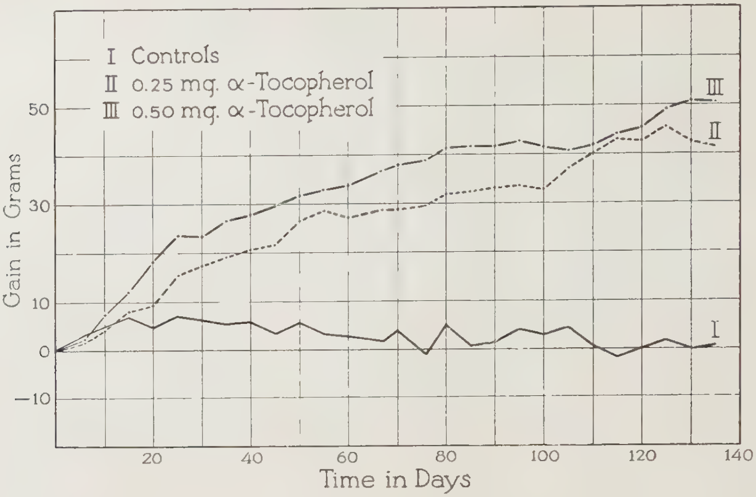


FIG. 1.

stimulation corresponds closely with that obtained in a previous experiment in this laboratory in which 1 mg alpha tocopherol was given for the same length of time (Table I).

Conclusions. Growth is stimulated in "plateaued" E-low female rats when their diet is supplemented by the daily administration of alpha tocopherol at the 0.25 and 0.50 mg levels. The growth corresponds approximately to that previously secured by the daily administration of 1 mg of alpha tocopherol.

TABLE I.
Growth-Promoting Action of Different Levels of Alpha Tocopherol.

Year	6 x weekly supplements	No. of rats	Plateauing wt, g	Period when supplements were fed, days	Gain in wt in 50 days
1938	80 mg ethyl laurate	3	228	195-245	0
	1 mg alpha tocopherol in 80 mg ethyl laurate	6	228	195-245	22
1940	80 mg ethyl laurate	6	237	153-203	5
	0.25 mg alpha tocopherol in 80 mg ethyl laurate	6	237	153-203	27
	0.50 mg alpha tocopherol in 80 mg ethyl laurate	6	237	153-203	32

11614

Successive Generations of Vitamin E-Low Rats.*

Gladys A. Emerson and Herbert M. Evans.

From the Institute of Experimental Biology, University of California, Berkeley, California.

This study was undertaken to determine the effects of continued vitamin E deficiency upon growth, general well being and reproductive behavior in successive generations of vitamin E-low rats.

Thirty rats from the stock colony were placed on the standard vitamin E-low diet 427† at 21 days of age. They were bred for a trial gestation at the first pro-oestrus which occurred after the 60th day of age. All animals in the group underwent a typical resorption gestation. Twenty-five rats of this group were re-bred and were given a single dose of 1.0 g of wheat germ oil. All cast litters but in 3 cases all the young were dead. They were allowed to suckle their young.‡ Throughout the study each litter of more than 6 young was reduced to that number. Fifty-six from a total of 130 young survived 30 or more days; they are designated as second generation E-low rats. All were paralyzed to some extent. The paralyses in some cases were flaccid—in others spastic. The picture other than the occurrence of paralyses included such phenomena as the existence of a square "boxy" body form, puffy face and corneal films. The 56 animals that survived recovered from external signs of the dystrophy and the females were then handled as the first

---

* Aided by grants from the Research Board and the Department of Agriculture of the University of California, the Rockefeller Foundation, and Merck and Company. The following materials were generously contributed: alpha-tocopherol by Merck and Company, Rahway, New Jersey, and wheat germ (from which the oil was prepared) by General Mills, Inc., Minneapolis, Minnesota. Assistance was rendered by the Works Progress Administration, Official Project No. 65-1-08-62, Unit A-5.

† Diet 427:

Casein, commercial, precipitated with HCl	27
Cornstarch (cooked)	35
Salts (McCollum 185)	4
Lard	22
Cod liver oil (Squibb's)	2
Brewer's yeast	10

The mixed diet without the cod liver oil is allowed to stand for 2 weeks at room temperature to permit the rancid substances of the lard to destroy incipient traces of vitamin E. The cod liver oil is added just before feeding.

‡ During the lactation period each female was given in addition to diet 427 1.0 g of brewers' yeast 6 times weekly.

generation animals, *i. e.*, they were allowed to undergo a resorption gestation and were given 1.0 g of wheat germ oil on the first day of their second gestation.

Of a total of 27 second generation E-low females bred, 26 cast litters (2 litters consisted of dead young) and 1 resorbed. Of a total of 134 young, 48 survived 30 or more days; these were the third generation of E-low animals. The clinical pictures presented were the same as for the young of the first generation mothers.

A total of 22 third generation females were bred resulting in 6 failed implantations, 1 resorption and 15 litters, 2 of which had only dead young. Of a total of 70 living young, 30 survived 30 or more days; these are designated as fourth generation E-low animals.

The fourth generation animals ovulated infrequently and only 7 females could be bred for the next generation. Two resorbed and a total of only 20 young were born, 6 of which survived 30 or more days.

One female of the fourth generation was given 0.15 cc of wheat germ oil daily from day 10 and was subsequently bred; 6 out of 6 young survived 30 days. The results are summarized in Table I.

The progressive debility of successive generations of E-low females is shown in Table II. The opening of the vagina and first oestrus appeared at more advanced ages in successive generations of vitamin E-low females. Growth was likewise affected as can be

TABLE I.
Reproduction and Lactation History of Vitamin E-low Female Rats.

Generation	No. of rats bred	No. of litters	No. of young suckled	No. of young surviving 30 days
1st	25	25	130	56
2nd	27	26	134	48
3rd	22	15	70	30
4th	7	5	20	6
4th (received 0.15 cc wheat germ oil daily)	1	1	6	6

TABLE II.
Progressive "Debility" of Successive Generations of E-low Females.

Generation	No. of rats	Avg wt 21 days	Avg wt 60 days	Avg age opening of vagina	Avg age at 1st oestrus
1	30	46.4	188	30*	35
2	30	40.1	159	42	51
3	24	39.3	137	47	57
4	6	32.5	125	55	63

*This is appreciably earlier than is usual in our colony.

seen from the average weight of 188 g at 60 days for the first generation when contrasted with 125 g for the fourth.

Growth and sexual physiology were studied in males of the second, third and fourth generations. The differences were not as marked as in the females but all showed the characteristic plateauing in weight. It is of interest to note, however, that 3 fourth generation E-low males were given 0.15 cc of wheat germ oil from day 10. At the age of 1 year the 3 animals receiving wheat germ oil averaged 110 g more in weight than did 8 untreated males of the same generation.

The males in each generation were offered oestrus females approximately twice monthly from the age of 4 months.

As can be seen from Table III, although the E-low males in all generations were sterile, the prophylactic administration of wheat germ oil prevented such changes when administered to 3 fourth generation males.

Note was made of the appearance of deficiency symptoms, other than sterility, in older males. (Table IV.) The fourth generation animals receiving wheat germ oil did not show these deficiency symptoms; they were also fertile, as has been mentioned. These facts are important because they appear to indicate that the observed progressive "debility" of the generations of vitamin E-low rats herein reported was due solely to inadequate E, adequacy of the diet in other respects being thus established.

TABLE III.
Sexual Physiology of Successive Generations of E-low Males.

Generation	No. of males	No. of offerings of oestrus females	No. of instances of plug	No. of instances of sperm	No. of young sired
2nd	27	384	147	0	0
3rd	15	273	117	0	0
4th	8	101	40	0	0
4th (received 0.15 cc wheat germ oil daily)	3	18	11	18	142

TABLE IV.
Deficiency Symptoms Other Than Sterility in E-low Male Rats.

Generation	No. of animals	Sparse, coarse or yellowish* fur Age in days	Stiff gait noted Age in days
2nd	27	194	277
3rd	15	121	178
4th	8	100	150

*The fur of black rats turned a reddish brown.

Summary. Four generations of vitamin E-low rats were observed as to growth and reproductive behavior. In the female there was a delay in maturity in each successive generation. Growth was also affected.

The males in each generation were sterile but fertility was maintained in the fourth generation by the prophylactic administration of wheat germ oil.

Coarse, sparse, yellowish fur and dystrophy that appear in rats maintained on vitamin E-low diets appeared progressively earlier in each successive generation.

11615

A Virus from Cases of Influenza-like Upper-respiratory Infection.

THOMAS P. MAGILL. (Introduced by Joseph C. Hinsey.)

From the Department of Bacteriology and Immunology, Cornell University Medical College, New York City.

During the past few years at least two groups of investigators[1,2] have reported cases of upper-respiratory infection of unknown etiology, that resembled true "epidemic influenza" in clinical respects but which differed in that the convalescent serums failed to show any increase in capacity to neutralize standard strains of the influenza virus. Two similar cases of influenza-like infection occurred among the workers in this laboratory in February, 1940. The serums obtained from these cases 4 weeks after the infection and those obtained either at the time of or 3 weeks before the onset of illness were tested against the PR8 strain of the virus of epidemic influenza. Neither of the convalescent serums showed any detectable increase in capacity to neutralize the usual 1000 lethal doses of the virus; and neither of them fixed complement in tests against antigens prepared in the usual manner[3] from mouse lung suspensions. This apparent lack of development of antibodies reactive against this standard strain of influenza virus seemed to indicate that the influenza-like infection of neither of the cases had been due to the virus of epidemic influenza.

---

[1] Reimann, H. A., and Stokes, J., Jr., *Tr. Assn. Am. Physn.*, 1939, **54**, 123.

[2] Stuart-Harris, C. H., Smith, W., and Andrewes, C. H., *Lancet*, 1940, **1**, 205.

[3] Francis, T., Jr., Magill, T. P., Rickard, E. R., and Beck, M. D., *Am. J. Pub. Health*, 1937, **27**, 1141.

TABLE I.

Neutralization Tests of Serums from 2 Cases of Influenza-like Infection Against the PR8 Strain of Influenza Virus and Against the *TM* Virus.

Serum		PR8 strain versus different dilutions of serum					*TM* virus versus different dilutions of serum				
Case	Time after onset	1-10	1-25	1-50	1-100	1-250	1-2	1-8	1-32	1-128	1-512
TM	Less than 12 hr	0	0	+	++	D	+++	+++	D	D	D
		0	0	++	+++	D	+++	+++	+++	D	D
		0	0	++	++++	D	+++	++++	+++	++++	D
	4 wk	0	0	+	D	D	0	0	0	+	++
		0	+	++	++	++++	0	0	0	+	+++
		0	++	++	+++	+++	0	0	0	++	+++
EJ	3 wk before	0	0	++	+++	D	+	++	D	D	D
		0	0	++	++	++++	++	+++	D	D	D
		0	0	0	++	+++	++	+++	++++	++++	D
	4 wk	0	0	+	++	D	0	0	+	+++	D
		0	0	++	+++	D	0	0	++	+++	D
		0	0	0	++	D	0	+	++	+++	++++

0—Mouse showed no pulmonary consolidation.
+, ++, +++, ++++—Degrees of pulmonary consolidation in mice that survived.
D—Mouse died; extensive consolidation of lung.

The throat washings from both patients when inoculated intranasally in ferrets under ether anesthesia evoked a fever of the type considered characteristic of influenza. The infectious agent from one of the cases was transmitted serially in ferrets, but even after 10 passages the lungs of infected animals showed no consolidation. The infection was transmitted by intranasal inoculation from ferrets to Swiss mice; in that species blue-gray areas of pulmonary consolidation were produced on the first passage. After 9 passages the virus increased sufficiently in virulence to kill some of the mice but although it has now been passed over 20 times it has not yet acquired the capacity of regularly killing all of the mice that are inoculated. The virus nature of this infectious agent is indicated by the fact that it passes through Berkefeld filters which retain ordinary bacteria and by the fact that it maintains its virulence for mice after storage for at least one month in 50% glycerol. In order to avoid circumlocution the term *TM* virus will be applied to this infectious agent.

Neutralization tests were made with the *TM* virus against the serums obtained from the 2 cases. The procedure was the same as that ordinarily employed;[3] however, the 10% suspensions of infected mouse lung that were used as antigen contained only about 10 infectious doses per 0.025 cc and did not regularly kill mice. For purposes of comparison a new series of neutralization tests against the PR8 strain of virus were made in which the test amount of virus was reduced from the usual 1000, to less than 10 lethal doses. The results of these experiments are summarized in Table I.

The data (Table I) show that even when tested against less than 10 lethal doses the convalescent serums of neither of the patients had any detectable increase in capacity to neutralize the PR8 strain of influenza virus. In contrast, the serums from both of the patients showed a significant increase in capacity to neutralize the *TM* virus. That virus had been isolated from case *TM* at the time of onset of infection and can be assumed to have been the etiologic agent of that case and probably also of case *EJ*. Thus, the results in Table I present an example in which the convalescent serums of patients who had recently recovered from an influenza-like infection showed an increase in capacity to neutralize the particular virus homologous to their infection but did not show an increase in capacity to neutralize a standard strain of the virus of epidemic influenza. The question of whether or not the *TM* virus is a strain of the virus of epidemic influenza that differs in antigenic properties from the PR8 strain or is an entirely unrelated virus has not been determined.

11616

Susceptibility of Mice to Intracerebral Inoculation of *C. Diphtheriae* and Diphtheria Toxin*

Martin Frobisher, Jr., and Elizabeth I. Parsons.

From the Department of Bacteriology, School of Hygiene and Public Health, Johns Hopkins University.

Hitherto, mice have been regarded as not highly susceptible to virulent diphtheria bacilli or to diphtheria toxin. The experiments of Kolle and Schlossberger,[1] Hippke,[2] Wolff,[3] Lewy,[4] and Zinnemann[5] showed that relatively large subcutaneous, intraperitoneal, and intravenous doses of virulent *C. diphtheriæ* or diphtheria toxin are required to produce illness or death of the mice, and frequently fail to do even this. However, a rather distinctive reaction indicating involvement of the central nervous system, has been observed frequently to follow such infections. The striking features of this reaction have been variously described as tic-like, or choreiform movements with hyperirritability and clonic convulsions. None of the workers referred to made use of the intracerebral method of inoculating mice; neither did any of them investigate the effect of cultures of *C. diphtheriæ* believed to be avirulent for guinea pigs, or of any of the diphtheroids.

The present series of experiments was prompted by the results of a preliminary test to determine the effect of intracerebral injections of broth cultures of rabbit-virulent *C. diphtheriæ* in mice as compared with subcutaneous and intraperitoneal injections of the same material.

Each of 5 strains, including the gravis, mitis and indeterminate types, was inoculated intracerebrally into 4 mice of different breeds, making 20 mice in all. For purposes of comparison the same strains, plus one other, were inoculated intraperitoneally and subcutaneously. Included were mice belonging to the Swiss, Sherman, C-57, and B. H. breeds. Of the 20 mice inoculated intracerebrally (0.03 cc) all died within 48 hours, while of 24 mice inoculated intraperitoneally (0.5 cc) 10 died and 14 survived, and of 24 inoculated subcutaneously

---

* These studies were aided by a grant from the W. K. Kellogg Foundation through the American Public Health Association.

[1] Kolle, W., and Schlossberger, H., *Z. f. Hyg.*, 1920, **90**, 193.

[2] Hippke, E., *Z. f. Hyg.*, 1922, **98**, 432.

[3] Wolff, E. K., *Virch. Arch.*, 1922, **238**, 237.

[4] Lewy, F. H., *Virch. Arch.*, 1922, **238**, 252.

[5] Zinnemann, K., *J. Path. and Bact.*, 1940, **50**, 243.

(0.5 cc), all but one survived. No evidence was seen indicating marked differences between the effectiveness of different types of diphtheria bacilli in killing the mice, nor did any one of the 4 breeds of mice seem to be much more susceptible than the others. After intracerebral inoculation, nearly all the mice showed the peculiar tic-like movements, frenzied activity, and prolonged clonic convulsions referred to above, but no such reactions were noted following subcutaneous or intraperitoneal inoculations.

In more extensive experiments, attempts were made to infect normal and antitoxin-injected mice by the intracerebral route, not only with strains of *C. diphtheriæ* of known virulence for rabbits and chicks[6] but with others of twice-proven avirulence for rabbits and chicks as well as with several strains of diphtheroids. As controls, 2 heat-killed cultures of virulent strains were included. The results of one group of such inoculations are summarized in Table I.

As additional controls, various organisms of entirely different genera (*E. coli, E. typhosa, N. intracellularis, S. aureus, D. pneumoniæ,* and others), and sterile broth were inoculated in a similar manner into a total of 63 mice. A number of the mice receiving these latter materials died, but none of them showed a reaction in any way comparable with that seen in mice receiving living *C. diphtheriæ*. There was no evidence of any nerve involvement.

TABLE I.
Effect of Various Organisms When Injected Intracerebrally into Mice.

Material injected	Result of rabbit and chick virulence test	Strains	Mice	Dead*	Ill and recovered	No visible reaction
C. diphtheriæ (no antitoxin)	Positive	7	48	46	1	1
C. diphtheriæ (antitoxin)‡	”	7	60	26†	17	17
C. diphtheriæ (heat-killed)	”	2	12	0	0	12
C. diphtheriæ‡ (no antitoxin)	Negative	13	18	8	9	1
C. xerosis	”	9	39	0	0	39
C. pseudodiphthericum	”	6	36	0	0	36

*Only deaths within 4 days, or after longer period only following typical spastic and frenzied seizures, are included here.

†8 of these died in a convulsive seizure within about an hour after the infective dose.

‡Antitoxin was given in doses of from 200 to 400 units intraperitoneally from 3 to 18 hours prior to the infective dose. In one instance phenol-free antitoxin was also mixed with the culture for 1 hour before injection intracerebrally. There seemed to be no special advantage in this.

---

[6] Frobisher, M., Jr., *Science*, 1940, **92**, 88.

It was observed that nearly all of the mice receiving virulent culture plus antitoxin were definitely ill and were affected, in most respects, much as were the unprotected mice. However, as noted in Table I, about half recovered from their illness and survived without further evidence of damage. Two weeks later these survivors were inoculated intracerebrally with a virulent strain of *C. diphtheriæ,* whereupon all died or showed the typical signs described above.

It is particularly important to note that the mice inoculated intracerebrally with *rabbit- and chick-avirulent* strains showed the same distinctive signs, although of a somewhat lesser intensity, as those seen in the mice inoculated with rabbit- and chick-virulent strains.

In order to verify these results, the virulence of 12 of the "avirulent" strains was retested in rabbits. When again found wholly avirulent, these strains were again inoculated intracerebrally into mice with essentially the same results as before. Of 68 mice inoculated, 12 died in 18 hours and 13 more within one week. All of the latter, as well as most of the mice that survived more than a week showed typical choreiform movements and occasionally clonic spasms and convulsions.

Thirty-six mice inoculated intraperitoneally with 0.5 cc of 48-hour broth cultures of 6 of the same strains showed no sign of illness of any sort.

Effect of Toxin. An experiment was carried out to determine the susceptibility of mice to intracerebral inoculations of sterile, toxic, culture-filtrate of *C. diphtheriæ.* A standardized and well seasoned lot of toxin was selected. The M.L.D. for guinea pigs was .0036 cc. Dilutions were so prepared that the dose finally received by the mice (.03 cc) would contain about 5, 1, and 0.2 M.L.D., respectively, after the diluted material was mixed with an equal part of sterile broth or antitoxin containing 50 units per cc. The mixtures stood at room temperature for about one hour before inoculation into the mice. The details of the experiment are shown in Table II.

TABLE II.
Effect of Intracerebral Inoculation of White Mice with Different Amounts of Diphtheria Toxin.

Guinea pig M.L.D.'s of toxin received by the mice	Mice	Dead	Ill and recovered	No visible reaction
5 (with antitoxin)	6	0	0	6
5 (without ”	6	6	0	0
1 (with ”	6	0	0	6
1 (without ”	6	4	2	0
0.2 (with ”	6	0	0	6
0.2 (without ”	6	0	0	6

The effect of the toxin was, in general, quite comparable with that of cultures of *C. diphtheriæ,* especially that of the largest dose. There were, however, no early deaths; indeed, no definite symptoms were noted before 48 hours had elapsed. The mice which received 5 M.L.D. then began to show the characteristic hyperirritability and hyperactivity observed in those infected with live cultures. The period of illness was, in general, greatly prolonged, but all of the unprotected mice which became ill eventually died. The mice receiving 1.0 M.L.D. became ill a day or two later than those receiving 5 M.L.D. Those receiving the smallest dose showed no ill effect. The antitoxin controls showed no reaction. It is probable that the toxin, in the toxin-antitoxin mixture, was completely neutralized before injection.

These results indicate that mice are much more susceptible to toxin introduced by the intracerebral route than to toxin introduced subcutaneously or intraperitoneally, since it has been found by earlier workers that doses of from 80 to 100,000 guinea-pig-M.L.D.'s were required to kill mice by the latter routes.[5] These doses are from 16 to 2000 times the largest dose (5 g.p. M.L.D.) used in the present experiments.

It would appear that, by using the intracerebral route of inoculation in mice, degrees of susceptibility to *C. diphtheriæ* and its toxin not hitherto suspected may be demonstrated.

From the above observations, it is evident that diphtheria toxin, when introduced intracerebrally into mice, produces a reaction which is similar in all respects to that resulting from the intracerebral inoculation of live, rabbit-virulent cultures such as are generally known to be toxigenic. Moreover, *the same type of reaction is produced by rabbit-avirulent strains which have never been shown to be toxigenic.* The implication is clear that the effect of the latter is due to the production of minute amounts of toxin which become manifest when elaborated in intimate contact with nervous tissue of the mouse.

It seems, therefore, that the term "avirulent" when applied to this group of organisms can be used only in a relative sense, and that there may be a closer biologic relationship between virulent and avirulent diphtheria bacilli, than has been believed to be the case. This method of inoculation may provide a new tool with which to study the rôle of rabbit-avirulent diphtheria organisms in the epidemiology of the disease.

Summary. These experiments have revealed four main facts: (a) White mice are highly susceptible to intracerebrally-injected 48-hour broth cultures of rabbit-virulent strains of *C. diphtheriæ;* 99% of the mice so inoculated dying within 10 days. (b) Mice are likewise susceptible, but in somewhat lesser degree, to similar injections of rabbit-*avirulent,* and hitherto supposedly harmless, strains of *C. diphtheriæ;* about 85% of the mice showing highly characteristic reactions within 10 days. (c) Mice receiving minute doses of toxin intracerebrally exhibit the same definitive and fatal results observed in the mice receiving the rabbit-virulent and rabbit-avirulent strains. (d) A number of cultures of diphtheroids *(C. xerosis* and *C. pseudodiphthericum)* were harmless for mice when injected as indicated above. Several other organisms have failed to produce any reactions in any way resembling those produced by *C. diphtheriæ* and diphtheria toxin.

11617

Comparative *in vitro* Study of Various Sulfanilamide Derivatives on Typhoid-Dysentery Organisms.

Charles H. Rammelkamp and Marjorie L. Jewell. (Introduced by Chester S. Keefer.)

From the Thorndike Memorial Laboratory, Second and Fourth Medical Services (Harvard), Boston City Hospital, and the Department of Medicine, Harvard Medical School, Boston, Mass.

Following the introduction of sulfathiazole by Fosbinder and Walter[1] it soon became evident that the thiazole derivatives exhibited bacteriostatic action against a number of organisms *in vitro.*[2-5] Previous experience in investigating infections of the urinary tract[6] due to *B. coli, B. lactis aerogenes* and *B. proteus* suggested that sulfathiazole might be an effective bacteriostatic agent against other Gram-negative organisms. The present study was undertaken to determine the comparative action *in vitro* of sulfanilamide, sulfapyridine, sulfamethylthiazole and sulfathiazole on representative organisms of the typhoid-dysentery group. The organisms used in this study were *Eberthella typhosa, Salmonella paratyphi, Salmonella schottmuelleri, Shigella dysenteriæ, Shigella paradysenteriæ*

---

[1] Fosbinder, R. J., and Walter, L. A., *J. Am. Chem. Soc.*, 1939, **61**, 2032.

[2] Long, P. H., and Bliss, E. H., Proc. Soc. Exp. Biol. and Med., 1940, **43**, 324.

[3] Rammelkamp, C. H., and Keefer, C. S., Proc. Soc. Exp. Biol. and Med., 1940, **43**, 664.

[4] Lawrence, C. A., Proc. Soc. Exp. Biol. and Med., 1940, **43**, 92.

[5] Hill, J. H., *J. Urol.*, 1940, **43**, 491.

[6] Rammelkamp, C. H., and Stoneburner, L. T., *New Eng. J. Med.*, in press.

(Flexner) and *Shigella sonnei.* Broth, urine and blood were used as media to study the effect of the various drugs.

Effect in Veal Infusion Broth. Methods. The chemical-broth media was prepared by adding powdered crystals to veal infusion broth so that the final concentration was 10 mg per 100 cc. The media was then sterilized by heating at 56° C for 2 hours. Sixteen-hour broth cultures of all organisms were used. To 5 cc of the control and broth containing the various drugs 0.5 cc broth dilution of the 16-hour culture was added. Incubation was carried out at 37°C for 24 hours. At specific intervals 0.5 cc of the culture was removed, proper dilutions made, and agar plates poured. These agar plates were then incubated for 24-48 hours and the colonies counted.

Results. Chart 1 shows in graphic form the results of these experiments in veal infusion peptone broth. The growth curves of the organisms using both a large and a small inoculum are recorded.* In general little bacteriostatic effect resulted when the original culture

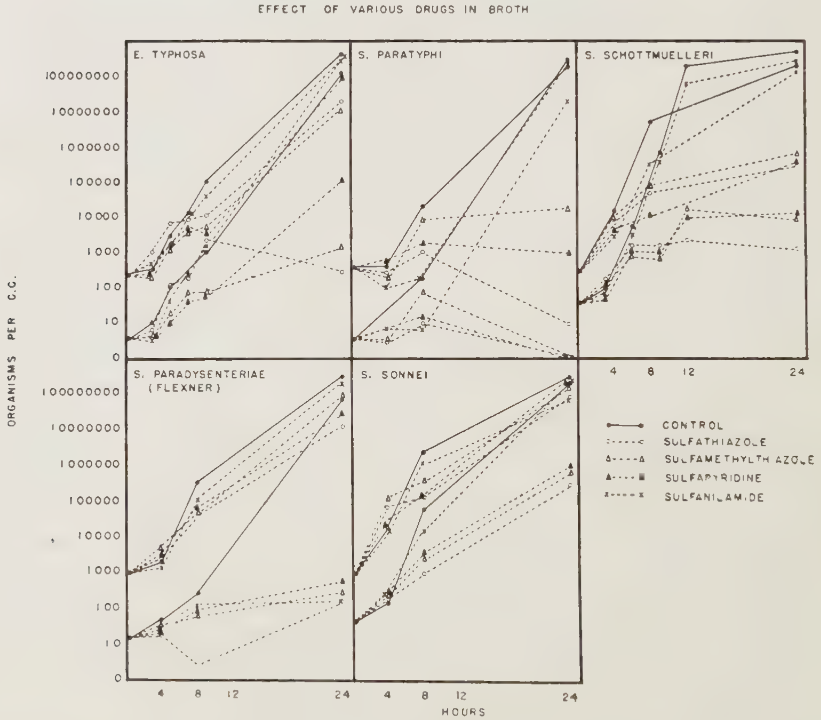


CHART 1.
The concentration of each drug is 10 mg per 100 cc.

---

* Growth curves using *Shigella dysenteriæ* (Shiga) are not reported because of failure of this organism to grow well in plain broth.

contained over 100 organisms per cubic centimeter. With the use of a large inoculum, sulfanilamide failed to inhibit the growth in any instance, whereas sulfathiazole, sulfamethylthiazole, and sulfapyridine showed some bacteriostatic action. In the experiments using *S. paratyphi* and *S. schottmuelleri* this inhibiting effect was striking.

The bacteriostatic effect becomes more apparent when small inocula are used; however, even then sulfanilamide showed no effect except in the case of *Shigella paradysenteriæ* (Flexner). Sulfathiazole usually caused more bacteriostasis than either sulfamethylthiazole or sulfapyridine, but the difference is not marked. In all curves there is an initial growth period of at least 4 hours before inhibition by the various drugs was noted.

Effect in Urine. Methods. The urine used in these experiments was obtained from pooled samples of urine from normal individuals

TABLE I.
Action of Various Drugs in Urine.

Organisms	Inoculum organisms per cc	Colonies per cc—24 hours				
		Control	Sulfanilamide	Sulfa-pyridine	Sulfa-methyl-thiazole	Sulfa-thiazole
E. typhosa	75,000	112,000,000	1,000	8	24	28
	1,400	312,000,000	300	48	56	72
	120	263,000,000	4	8	0	0
S. paratyphi	6,300	115,000,000	1,510,000	4	2,400	0
	1,780	458,000,000	29,600	12	0	8
	100	6,640,000	418	9	2	0
	4	25,000	10	0	0	0
S. schottmuelleri	42,000	117,000,000	4,520,000	580	4	280
	1,500	87,000,000	2,750,000	104	40	14
	300	296,000,000	600,000	528	328	404
	50	112,000,000	4,000	12	4	0
	30	68,000,000	100,000	0	0	0
S. paradysenteriæ (Flexner)	15,000	98,000,000	20	8	0	8
	5,400	68,000,000	40	4	8	0
	830	125,000,000	68,000	0	0	0
	66	2,000,000	4	4	0	0
S. sonnei	21,000	71,000,000	20,000	200	12	0
	5,100	169,000,000	100,000	340	604	900
	540	260,000	20	4	8	0
	60	180,000,000	40	4	24	4
S. dysenteriæ	42,000	1,200,000	1,000	28	200	12
	5,200	128,000,000	78,000,000	14,400	1,200	72
	820	134,000,000	72,000,000	500	160	400
	34	330,000,000	520,000,000	320	32	0

The final concentration of each drug was 10 mg per 100 cc.

and adjusted to pH 7.0. The various drugs were added so that the final concentration was 10 mg per 100 cc. The urine was then passed through a Berkefeld filter. All dilutions of the cultures were made in 0.85% sodium chloride. 0.5 cc of the proper dilution was then added to 5 cc of the control and the urine containing the various drugs. Growth curves were then done following the technic used above.

Results. In Table I the results of the *in vitro* studies using urine as the media are recorded. Since the growth curves are similar in character to those in Chart 1, only the number of organisms present after 24 hours' incubation are recorded. Again, experiments using large and small inocula are included for each organism. In the control cultures, growth was maximal in most instances. Sulfanilamide caused some inhibition of growth in the majority of experiments, however, the urine was never sterilized after 24 hours of incubation. The other drugs caused not only inhibition, but also some bactericidal action. Sulfathiazole and sulfamethylthiazole were only slightly superior to sulfapyridine.

Effect in Blood. Methods. Because relatively large numbers of typhoid-dysentery organisms are killed in normal defibrinated blood, the media was prepared as follows. Venous blood was obtained from normal individuals, defibrinated, and the serum separated by centrifugalization. The serum was then placed in a water bath at 57°C for 60 minutes. The red cells were washed in saline and added to the treated

TABLE II.
Action of Various Drugs in Blood.

		Colonies per cc—24 hours				
Organism	Inoculum organisms per cc	Control	Sulfanilamide	Sulfa-pyridine	Sulfa-methyl-thiazole	Sulfa-thiazole
E. typhosa	50,000	268,000,000	55,000,000	340,000	1,520,000	290,000
	1,290	50,000,000	12,000	4	24	24
S. paratyphi	7,000	180,000,000	3,000,000	164	120	40
S. schott-muelleri	6,100	85,000,000	104,000,000	1,040	600	264
	400	50,000,000	72,000,000	36	4	48
S. para dysenteriæ (Flexner)	41,500	23,000,000	12	28	4	4
	300	19,000,000	15,000	48	148	100
S. sonnei	31,700	73,000,000	44,000,000	130	124	84
	2,300	122,000,000	30,000	900	36	24
S. dysenteriæ	6,400	44,000,000	80	8	8	8
	200	4,000,000	12,000,000	60	2	0

The final concentration of each drug was 10 mg per 100 cc.

serum in a concentration of about 10%. To 3 cc of this complement free blood, 0.6 cc of a 70 mg/100 cc saline solution of the various drugs and 0.6 cc of the proper saline dilution of the 16-hour broth cultures were added. The final concentration of the drug in the culture was 10 mg/100 cc. Growth curves were then followed, using a technic similar to that outlined above.

Results. Again, only the number of organisms present after 24 hours' incubation is recorded in Table II, however, the growth curves showed that the inhibiting effect, when present, did not become manifest until after 4 hours' incubation. Sulfanilamide usually caused some inhibition of growth. Sulfapyridine, sulfamethylthiazole and sulfathiazole all caused bacteriostasis in every experiment and, indeed, some killing of the organisms was noted.

Discussion. The results of the *in vitro* studies in broth confirm in part the experiments reported by Lawrence.[7] He found that sulfathiazole and sulfamethylthiazole were somewhat more effective than sulfanilamide and sulfapyridine in dextrose broth against *E. typhosa, Shigella dysenteriæ, S. paratyphi* and *S. schottmuelleri.* In his experiments, the largest inoculum used was 185 organisms per cc. Although he reported some degree of bacteriostasis, in only one instance was there bactericidal action as well.

When urine or blood was used as the culture media, bactericidal as well as bacteriostatic effects were demonstrable. The difference between sulfathiazole, sulfamethylthiazole and sulfapyridine on the test organisms used in blood and urine is not marked, however, all 3 drugs showed a greater effect than sulfanilamide.

Conclusions. The effect of sulfathiazole, sulfamethylthiazole, sulfapyridine and sulfanilamide in concentrations of 10 mg per 100 cc on large and small inocula of *E. typhosa, S. paratyphi, S. schottmuelleri, Shigella dysenteriæ, Shigella paradysenteriæ* and *Shigella sonnei* in veal infusion peptone broth, urine and blood was studied. It was found that sulfanilamide had little bacteriostatic effect in broth cultures, whereas, if a small inoculum is used, sulfathiazole, sulfamethylthiazole and sulfapyridine showed a bacteriostatic action. In urine and blood, sulfanilamide usually caused some inhibition of growth. The action of sulfathiazole, sulfamethylthiazole and sulfapyridine in urine and blood was not only bacteriostatic but was often bactericidal. Sulfathiazole was only slightly superior to sulfamethylthiazole and sulfapyridine when urine and blood were used as media.

Of the 6 organisms studied, *S. paradysenteriæ* (Flexner) appears to be most susceptible to the action of the sulfonamide drugs. No further conclusions as to the comparative susceptibility of the strains used in this study seems justified.

---

[7] Lawrence, C. A., PROC. SOC. EXP. BIOL. AND MED., 1940, **44**, 162.

We are indebted to Squibb Institute for Medical Research for the sulfathiazole and to Winthrop Chemical Company for the sulfamethylthiazole used in these experiments.

We wish to thank Miss Eleanor M. Fleming for her technical assistance.

11618

Effect of Ether-Soluble Fraction of Bile on Hepatic Glycogen Storage.

R. W. VANCE, E. SNAPP AND A. C. IVY.

From the Department of Physiology and Pharmacology, Northwestern University Medical School, Chicago.

Mizuta and Ikegami[1] have reported the presence in cattle bile of an ether-soluble substance which promotes glycogen formation in the liver. We decided to repeat their experiments because the data presented are not convincing and the concept may possess significance.

Methods. We have conformed as closely as possible to the general experimental plan of the Japanese observers. Young adult rabbits were fed our standard stock diet for 2 weeks. They were then placed in a stock in a wire cage to prevent coprophagia and fasted for 72 hours. The animals were then given 2 or 4 g of glucose per kilo body weight slowly intravenously (10 minutes) and 30 minutes later were given the bile extract similarly. 2.5 hours later, the animals were given pentobarbital intravenously, which induces anesthesia immediately. The liver was frozen *in situ* with CO_2 within a minute. The frozen liver was crushed to powder and a weighed portion placed in 30% KOH, digested and analyzed for glycogen by Somogyi's method. The results were calculated and expressed as the percent of glycogen in the powder.

The extracts were made from freshly frozen cattle bile. One extract, A, was made by rendering the thawed bile slightly alkaline (pH 8.0), evaporating to dryness *in vacuo* at a low temperature for 24 hours, and extracting the powder with ether and removing the ether *in vacuo*. A second extract, B, was made by slightly acidifying the thawed bile (pH 4.0) with phosphoric acid, steam distilling for

---

[1] Mizuta, N., and Ikegami, Y., *Jap. J. Gastroenterol.*, 1937, **9**, 258.

6 hours, the distillate being extracted with ether and the ether removed *in vacuo*. A third extract, C, was made as in the first extract, A, except the thawed bile was slightly acidified (pH 4.0) with phosphoric acid.

That amount of extract equivalent to 6 cc of the fresh bile was injected slowly intravenously.

Results. The liver glycogen content of 10 control rabbits, which were fasted, treated with 2 g of glucose per kilo but were not given the bile extracts, averaged 1.37%, range 0.11 to 2.93%. The liver glycogen content of 5 rabbits which received bile extract A averaged 1.16%, range 0.29 to 1.78%; of 6 rabbits which received extract B averaged 1.22%, range 0.15 to 1.99%; of 10 rabbits which received extract C averaged 1.44%, range 0.13 to 2.03%. None of these differences are significant. The average liver glycogen of the 21 rabbits receiving the bile extracts was 1.31%, which compares very favorably with the average of 1.37% for the controls.

Using extract C on 11 rabbits which were given 4 g of glucose per kilo instead of 2 g, the average liver glycogen found was 2.01%, range 0.96 to 2.97%. The average liver glycogen of 8 control rabbits receiving the same amount of glucose was 3.0%, range 1.6 to 3.6%. This difference is statistically significant, indicating that the extract interfered with the deposition of glycogen; however, we doubt its physiologic significance. In 5 rabbits the ether extract of dried but otherwise unmodified cattle bile had no effect on glycogen deposition.

We were, therefore, unable to confirm the report that bile contains a substance which favors glycogen deposition in the liver.

11619

Effect of Inhalation of High Oxygen Concentrations, With and Without Carbon Dioxide, on the Electrocardiogram.

ALVAN L. BARACH AND ALFRED STEINER.

From the Research Service, First Division, Welfare Hospital, Department of Hospitals, City of New York, the Department of Medicine, College of Physicians and Surgeons, Columbia University, and the Presbyterian Hospital, New York City.

In another communication[1] it was shown that the addition of 2 to 3% carbon dioxide to low oxygen mixture reversed the electrocardiographic changes of induced anoxemia in the majority of patients with heart disease and in some normal individuals. The physiological basis for the trial of carbon dioxide was the existence of alkalosis at the termination of a 20-minute period of oxygen want. Since it had been recognized that alkalosis stimulates contraction of smooth muscle and constriction of the capillary bed, and that administration of carbon dioxide has an opposite influence, the hypothesis was presented that these factors might play a rôle in the regulation of the coronary circulation. It was also recognized that breathing even small percentages of carbon dioxide increased the arterial oxygen saturation and facilitates the unloading of oxygen into the tissues, factors which will be presented in a fuller report.

---

[1] Barach, A. L., and Steiner, A., to be published.

In this study the effect on the electrocardiogram of inhalation of 100% oxygen and of 97 to 96% oxygen with 3 to 4% carbon dioxide is presented. Observations were made on 26 patients in whom primary heart disease was present, or in whom cardiac function was affected by pulmonary or other disease. In addition, 6 normal and 2 patients with chronic arthritis were similarly studied.

Methods. The method of administration of the gas employed has been previously described.[2] After the control electrocardiogram was obtained the patient was given 100% oxygen for 20 minutes; after a rest period of 10 minutes, oxygen with carbon dioxide was given for a similar period. In the second test of case 1, oxygen was administered for one hour.

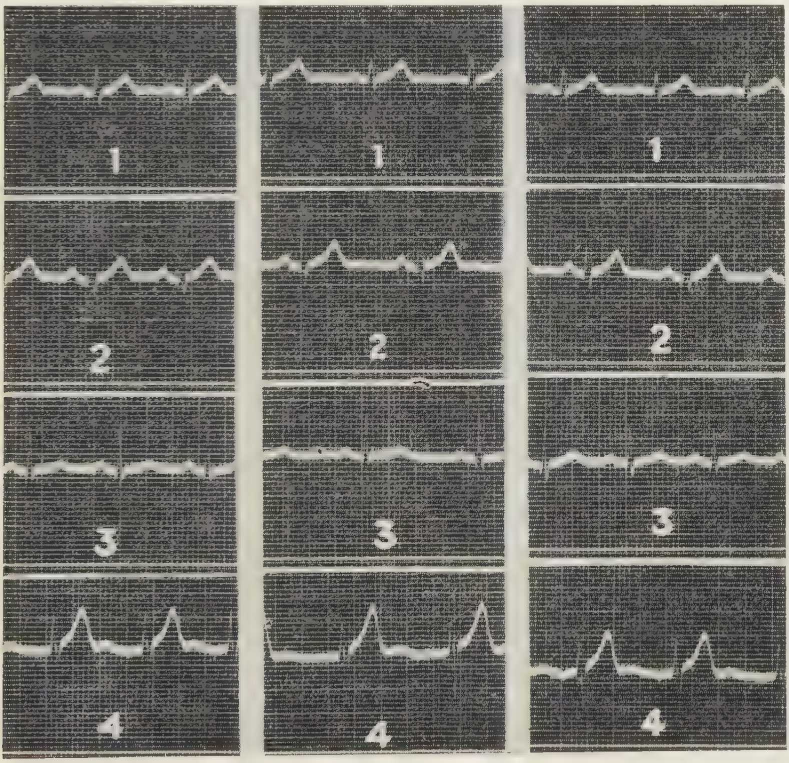
Results. Of the 26 cases in the cardiac group, 20 responded to inhalation of either 100% oxygen, or 97% to 96% oxygen and 3 to 4% carbon dioxide, with elevation of the T wave, in one or more of the 4 leads, less inversion of the T wave, or a T wave previously diphasic becoming upright. In 4 cases there was no change after inhalation of either oxygen or oxygen with carbon dioxide. In one case T_1 was very slightly lower after both tests and in another T_3 was very slightly lower after oxygen and very slightly higher after oxygen with carbon dioxide.

Of 20 cases in which the electrocardiographic changes due to 100% oxygen were compared with those due to oxygen plus carbon dioxide, there were 7 in which the T wave elevation was higher with 100% oxygen, one in which the oxygen plus carbon dioxide mixture was very slightly higher, and 12 in which the result was approximately the same. In the 12 instances in which the elevation of the T wave was noted as being equal after the two tests, the impression was frequently gained that there was very slight lowering of the T wave when carbon dioxide was added to the oxygen mixture, as contrasted to the effect of 100% oxygen; it was believed, however, that changes of a magnitude of 0.25 mm were too small to be considered significant. The increase in height of the T wave ranged from 0.5 to 2.0 mm; occurring more often in lead 4 than in the other leads.

---

[2] Levy, R. L., Barach, A. L., and Bruenn, H. G., *Am. Heart J.*, 1938, **15**, 187.

In the group of 2 cases of chronic arthritis and 6 normal individuals there was no change due to inhalation of 100% oxygen in 6, and elevation of the T wave in 2. In 5 of this group there was no change due to inhalation of the oxygen-carbon dioxide mixture; in 4, there was elevation of the T wave in 1, and lowering in 2. The changes were less frequent in the control group, but in one instance illustrated in the text (Electrocardiographic Picture No. 1) there was definite elevation of the T wave in all 4 leads; these changes were slightly less marked with the oxygen-carbon dioxide mixture. An

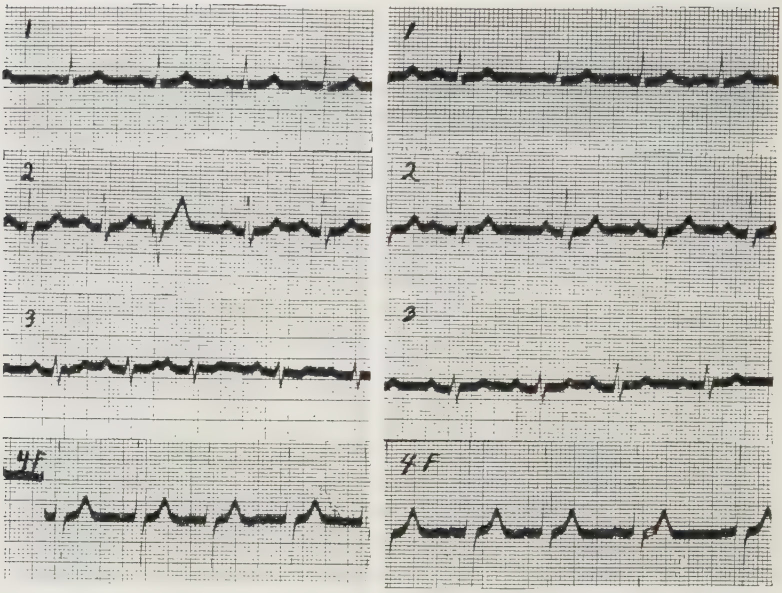


ELECTROCARDIOGRAPHIC PICTURE No. 1.

A. Electrocardiogram of normal individual breathing air.

B. After breathing 100% oxygen for twenty minutes, T_1, T_2, T_3, T_4 higher.

C. After breathing 96% oxygen and 4% CO_2 for twenty minutes, changes are similar to B, although slightly less marked.



ELECTROCARDIOGRAPHIC PICTURE NO. 2.

A. Electrocardiogram of patient with coronary sclerosis breathing air.

B. After inhalation of 97% oxygen and 3% carbon dioxide for twenty minutes, T_2 and T_4 are higher, and T_3 previously diphasic is upright.

illustration of the effect of 97% oxygen and 3% carbon dioxide is shown in the accompanying illustration (Electrocardiographic Picture No. 2). It will be observed that T_2 is higher, T_3 which was diphasic has become upright, and T_4 is higher.

According to Wilson, *et al.*,[3] and MacLeod[4] the T wave represents that part of the cardiac cycle in which heart muscle is in the main regressing from the active state, during which products of metabolic activity are being eliminated. According to MacLeod, when this recovery period is prolonged, the T wave is relatively lower; conversely, shortening of the recovery period is represented by a more elevated T wave. If we apply these concepts to our findings an explanation is seemingly afforded. The inhalation of low oxygen mixtures prolongs the recovery period and results in flattening of the T wave; inhalation of 100% oxygen shortens the recovery period in some cases and is followed by elevation of the T wave. When alkalosis is induced by acute anoxia, the administration of small doses of carbon dioxide tends to counteract the T wave depression induced by acute anoxia: in the absence of alkalosis in the resting individual the addition of 3 to 4% carbon dioxide to a high oxygen mixture may result in less marked elevation of the T wave than that induced by 100% oxygen. This would suggest, according to MacLeod's studies, that increasing the CO_2 tension in the blood beyond that normally present may prolong the recovery period of the cardiac muscle. Additional studies will be made on the comparative effects of 1 to 2% carbon dioxide, with the remainder oxygen.

---

[3] Wilson, F. N., MacLeod, A. G., and Barker, P. S., *Currents of Action and of Injury*, Univ. of Michigan Studies, Scientific Series X, 1933.

[4] MacLeod, A. G., *Am. Heart J.*, 1938, **15**, 165; *ibid.*, 1938, **15**, 402; also, personal communication.

It is pertinent to mention that although 50% oxygen has been shown to relieve anginal pain,[5, 6] more striking clearing of pain resulting from acute coronary occlusion has been recently reported as result of inhalation of 100% oxygen.[7]

Summary. Inhalation of 100% oxygen has been shown to result in the elevation of the T wave in the electrocardiogram in 20 of 26 cardiac cases, and in 2 of a group of 8 normal and miscellaneous cases. When 97 or 96% oxygen with 3 or 4% carbon dioxide was tested, similar electrocardiographic changes were observed although the degree of change was frequently less marked; thus, in 7 of 20 cardiac cases the T wave was distinctly more elevated after inhalation of 100% oxygen than after the oxygen-carbon dioxide mixture. The significance of these observations is discussed. It seems likely that inhalation of 100% oxygen shortens the recovery period of cardiac muscle.

---

[5] Rizer, R. L., *Minnesota Med.*, 1929, **12**, 506.

[6] Barach, A. L., *Ann. Int. Med.*, 1931, **5**, 428.

[7] Boland, E. W., *J. A. M. A.*, 1940, **114**, 1512.

11620

True Uric Acid in Hyperuricemia of Pre-Eclampsia and Eclampsia.*

N. K. SCHAFFER AND H. J. STANDER. (Introduced by Vincent du Vigneaud.)

From the Department of Obstetrics and Gynecology, New York Hospital and Cornell University Medical College.

The determination of uric acid in blood with the aid of uricase by Blauch and Koch[1] partitions the ordinary value, obtained by direct reduction of phosphotungstate, into true uric acid and non-uric acid fractions. Thus they report that for normal blood only two-thirds (about 2 mg %) of the ordinary value consists of true uric acid. A rise in blood uric acid is a well established finding in pre-eclampsia and eclampsia.[2] We have applied the uricase method to the hyperuricemia of these pregnancy toxemias in order to determine how much of it is contributed by true uric acid and by non-specific material.

Methods. Blood uric acid was determined before and after uricase treatment according to Blauch and Koch, except that the color was developed by the Folin method.[3]

Results. Two cases of pre-eclampsia and 3 cases of eclampsia were studied (Table I). It is apparent that the blood uric acid in these conditions is practically all accounted for by true uric acid.

Summary. Using the uricase method for the determination of blood uric acid from 89 to 96% of the hyperuricemia of pre-eclampsia and eclampsia consists of true uric acid.

TABLE I.
True Blood Uric Acid in Pre-eclampsia and Eclampsia. Values in mg %.

Case		Before uricase treatment	After uricase treatment	True uric acid	% true uric acid
M.	Pre-eclampsia	4.85	.27	4.58	94
S.	”	7.58	.39	7.19	95
P.	Eclampsia	5.45	.58	4.87	89
K.	”	6.11	.57	5.54	91
H.	”	9.88	.42	9.46	96

---

* Supported by a grant from the John and Mary R. Markle Foundation.

[1] Blauch, M. B., and Koch, F. C., *J. Biol. Chem.*, 1939, **130**, 443.

[2] Cadden, J. F., and Stander, H. J., *A. J. Obst. and Gynec.*, 1939, **37**, 37.

[3] Folin, O., *J. Biol. Chem.*, 1933, **101**, 111; 1934, **106**, 311.

11621 P

Exchange of Eyes Between Adult Hosts of *Amblystoma punctatum* and *Triturus viridescens*.*

L. S. Stone and F. S. Ellison.

From the Department of Anatomy, Yale University School of Medicine.

It has been shown by one of the authors that functional eyes can be successfully exchanged between the larvae of 2 closely allied species, *Amblystoma punctatum* and *A. tigrinum*.[1] Since we have also demonstrated that homoplastic eye grafts of adult *A. punctatum*[2] and *Triturus viridescens*[3, 4, 5] can be made with return of vision, it was of interest to see what success could be obtained in these 2 very different species if their eyes were exchanged. In the present experiments the right eyes were exchanged between 34 pairs of animals. The Amblystoma hosts were young adults around 10 to 11 cm in length. The Triturus animals were about 9 cm in length. The eyes were studied daily while living and the hosts were preserved from 14 to 141 days after operation for histological studies.

In Amblystoma eyes on Triturus hosts return of circulation was observed as early as the fifth day. In the majority of cases it was recorded during the first 2 weeks. In Triturus grafts it was first seen on the sixth day and recorded in one-half of the cases during the first 2 weeks. In some cases temporary opacity in the cornea in both groups delayed the observation of the first visible signs of circulation. In some Triturus eyes the opacity, when it appeared, lasted for many days. Corneal reflex was obtained in Amblystoma grafts after the first month. This appeared to be present in a few Triturus eyes also.

The experiments demonstrated that Amblystoma eyes were far better adapted to their new hosts than the Triturus grafts. Although there was sloughing and resorption of both types of grafts the Triturus eyes on Amblystoma if kept long enough beyond the sixth or seventh week ended in this way.

In the Triturus iris the pigment pattern of black and yellow broke

---

* Aided by grants from the John and Mary R. Markle Foundation and the Fluid Research Fund of Yale University School of Medicine.

[1] Stone, L. S., *J. Exp. Zool.*, 1930, **55**, 193.

[2] Stone, L. S., and Cole, C. H., Proc. Soc. Exp. Biol. and Med., 1931, **29**, 176.

[3] Stone, L. S., and Ussher, N. T., Proc. Soc. Exp. Biol. and Med., 1927, **25**, 213.

[4] Stone, L. S., Zaur, I. S., and Farthing, T. E., Proc. Soc. Exp. Biol. and Med., 1934, **31**, 1082.

[5] Stone, L. S., and Chace, R. R., Proc. Soc. Exp. Biol. and Med., 1937, **36**, 830.

down during the first 2 weeks, just as it does when homoplastic grafts were made.[4] An attempt to repair this pattern was later made but subsequent degeneration usually overtook it. In the Amblystoma eye the iris, which is brownish black with a few golden spots, usually changed very little.

The pupil of the normal Triturus eye is rather large and oval in shape. It does not react to light.[4] After operation it decreased maximally in size as in homoplastic grafts. In the present experiments it never recovered. In the adult Amblystoma eye there is a marked pupillary light reflex. In most of these transplanted eyes the pupillary reflex continued after the operation. In some there was a temporary resting stage of dilation. In one case this remained as a permanent condition although the pupil did give a response to light. In a few cases between the third and twelfth days after operation the pupil in the resting stage was much smaller than normal. It remained in this condition for about a week before it recovered. No pathology in the iris or ciliary body was found histologically in specimens killed during such periods.

The retinal picture obtained from histological studies showed early stages of degeneration in both types of eyes, such as exist in homoplastic grafts.[2, 4] In the Triturus transplants on the twentieth to twenty-fifth day the degenerative processes were continuing further. At this time in homoplastic grafts the more resistant ciliary region of the retina ordinarily is regenerating a new retina. This is inhibited when the eye is in the Amblystoma host. Blood vessels begin to invade all of the deeper tissues and progressive resorption takes place.

Amblystoma eyes on Triturus hosts reacted entirely differently. They followed more closely the course of homoplastic grafts. The undegenerated ciliary region of the retina began regenerating a new retina during the third week. Although some eyes showed slower regeneration than others a new retina began differentiation into its various layers during the second month. As early as the forty-first day a completely regenerated optic nerve was seen extending from the graft to the chiasma of the new host.

The regenerated retina was thinner than the normal. The ganglion layer had less cells than normal, for there were spots in it devoid of cells. The inner nuclear layer also appeared to have less cells than the normal retina. The rod and cone cell layer appeared normal. As late as 141 days after operation there were no signs that the Amblystoma eye on a Triturus host was undergoing any degeneration.

No tests for vision were made in this group of animals. Such tests will be made in further experiments when a larger group of animals is available.

11622 P

Origin of the Lens by Induction in the Salamander *Amblystoma punctatum*.*

L. S. Stone and F. L. Dinnean.

From the Department of Anatomy, Yale University School of Medicine.

Harrison[1] demonstrated in *Amblystoma punctatum* that lens development was suppressed if the eye rudiment was excised just before or immediately after the closure of the neural folds, but that the same presumptive lens ectoderm would form lens tissue if grafted to other parts of the head. This indicated that the lens was established very early even though a prolonged association with the eye insured better development. He also showed that at this time body and head ectoderm was not transformed into lens if grafted over these eye centers. In stages after the closure of the neural folds this was corroborated by Beckwith[2] and Stone and Dinnean.[3]

The present report involving 60 experiments demonstrates lens induction by the eye in this salamander and shows how the various lens-eye relationships in cyclopia can be explained on this basis.

The presumptive lens forming region in the open neural plate (Harrison stages 15 and 16) was completely excised on the right side and replaced by ectoderm which was taken from the ventral pole of Harrison stage 12 to 13, previously stained in Nile-blue sulphate. The blue ectoderm later covered the entire side of the head including the eye. The latter induced a lens in the grafted ectoderm. In histological sections the lens contained the blue dye, therefore leaving no doubt as to its origin.

In one group of experiments in this series the prechordal substrate under the neural plate (Harrison stage 15) was excised in the manner described by Mangold[4] and Adelmann,[5] while in another group, eggs (Harrison stages 8 and 9—when involution of blastopore begins) were placed in lithium chloride (0.4% solution) for 24 hours.

---

* Aided by grants from the John and Mary R. Markle Foundation and the Fluid Research Fund of Yale University.

[1] Harrison, R. G., Proc. Soc. Exp. Biol. and Med., 1920, **17**, 199.

[2] Beckwith, C. J., *J. Exp. Zool.*, 1927, **49**, 217.

[3] Stone, L. S., and Dinnean, F. L., *J. Exp. Zool.*, 1940, **83**, 95.

[4] Mangold, O., *Ergebn. d. Biol.*, 1931, **7**, 365.

[5] Adelmann, H. B., *J. Exp. Zool.*, 1930, **57**, 223.

The animals were killed a few days later in Harrison stages 39 to 41—when the lens is well defined. Both experiments as Adelmann[5, 6] has shown in Amblystoma produced many head deformities, particularly in the eye regions. All degrees of fusion of the eyes resulted and in a few animals cyclopia completa was obtained.

In some cases there were no eyes and when this occurred, there were no lenses. Some specimens possessed a poorly formed eye with a lens, while on the other side lens and eye development were completely suppressed. In 2 cases there were 2 fused flattened optic plates with much mesoderm lying between them and the overlying ectoderm. In these there was no lens formation. The intervening mesoderm apparently prevented lens induction. In other specimens fusion of the eyes was found in all degrees. In some of these a single eye of 2 fused components possessed a single elongated lens with 2 separated fiber forming centers. When the complete cyclopian condition existed, the lens was large with a single fiber forming pole.

The similarity in the degree of fusion between the eyes and the lenses indicates in the light of these experiments that the fiber forming pole (the center for lens formation) was a direct result of the inducing eye center. The experiments also demonstrate that so far as induction of the lens by the eye is concerned Amblystoma falls in line with that of many other amphibians. This answers the question which Spemann[7] raised regarding this species.

---

[6] Adelmann, H. B., *J. Exp. Zool.*, 1934, **67**, 217.

[7] Spemann, H., 1938, *Embryonic Development and Induction,* Yale University Press, 40.

11623

Antipressor Effect of Normal Blood in Experimental Hypertension.

Gustave Freeman. (Introduced by G. F. Dick.)

From the Department of Medicine, University of Chicago.

Goldblatt, Lynch, Hanzal and Summerville[1] devised a successful method for producing permanent hypertension by clamping renal arteries in dogs. Many substances like the nitrites and thiocyanates have been used from time to time to reduce blood pressure in man and animals. Extracts of various tissues have been tried also, but none of these have proven to be physiologically specific for hypertension. Experiments are devised in this study to investigate an antipressor substance in blood of normal dogs in relationship to the hypertension of Goldblatt, *et al.*

Method. Initial blood pressures were taken on all dogs without anesthesia by puncture of the femoral arteries. Hypertensive dogs were prepared by the Goldblatt method. Transfusions were made by drawing blood from the femoral arteries of the donors into warm syringes of 100 cc capacity containing sodium citrate solution and injecting it immediately into the prepared foreleg veins of the recipients. Blood pressures were recorded on a kymograph using a cannula between the femoral artery and a mercury manometer. In several experiments the donors' blood pressures were recorded also by the same method. Experiments were carried out using sodium pentobarbital anesthesia. Dogs were used only once.

Results. Two kinds of curves were obtained having distinctly different characteristics (Graph 1). Besides the experimentally produced reduction in the pressure, precipitous falls occurred frequently during the period of transfusion and recovered immediately regardless of the type of experiment or subsequent course of the blood pressure. Donor and recipient bloods were cross-matched and usually showed weak clumping but no true agglutination. Both serum and plasma were used in the cross-matching. No correlation was observed between the results of cross-matching and the type or incidence of pressure changes occurring as a result of transfusion. Such blood pressure changes always occurred within the period of transfusion and the baseline was regained immediately afterwards. The falls were precipitous and occasionally reached shock levels.

---

[1] Goldblatt, Harry, Lynch, James, Hanzal, R. F., and Summerville, W. W., *J. Exp. Med.*, 1934, **59**, 347.

GRAPH I

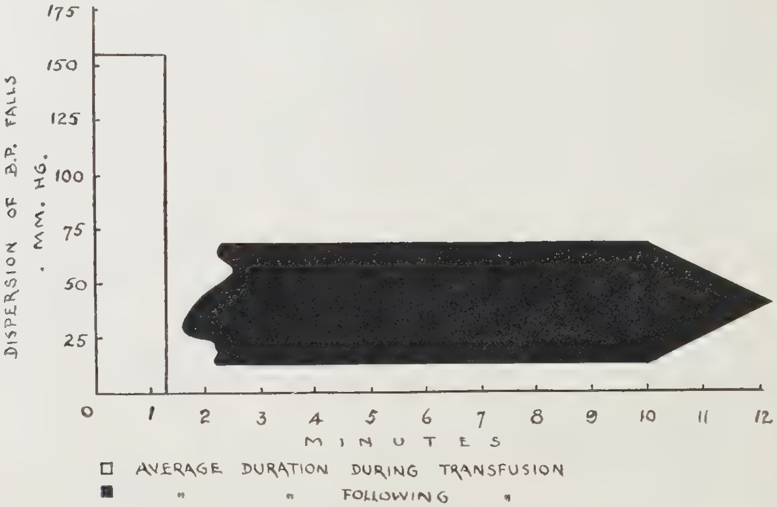


TABLE I.
Normal Donor and Hypertensive Recipient.

No.	Quantity blood (cc)	Initial B.P. (mm Hg)	Transfusion fall		Duration trans-fusion		Prolonged fall		
			Depth	Duration			Depth	Duration	%
					min	sec			
2	180	197	0		4	0	68	9 min+	35
3	100	187	0		1	10	56	9 '' +	30
4	160	182	0		2	0	29	15 '' +	16
5	200	200	0		3	2	69	6 ''	35
8	100	162	155	2 min 5 sec	4	0	11	12 '' +	6
9	25*	179	0		1	0	46	31 '' +	26
13	110	188	31	3 '' 20 ''	3	30	32	†7 '' +	17

*A mixture of renal venous and femoral arterial blood.
†The pressure was still falling when a technical accident interfered.

These changes were easily differentiated from the predictable, prolonged depressions that persisted after cessation of the transfusions. Graph 1 demonstrates the scattering of the extent against the average duration of this "incompatibility" effect as compared to the prolonged blood pressure changes that occurred within experimental expectations.

Table I contains the results of transfusing hypertensive dogs with the blood of normal donors. Seven experiments were done.

Experiments were usually stopped when the recipients' pressures began to rise to the initial levels.

An attempt was made to stimulate the production of antipressor substance by the normal kidneys of donor dogs in all the above experiments except Nos. 8 and 9. In order to do so donors were given

TABLE II.
Normal Donor and Normal Recipient.

No.	Quantity blood (cc)	Initial B.P. (mm Hg)	Transfusion fall Depth	Transfusion fall Duration min	Transfusion fall Duration sec	Duration transfusion min	Duration transfusion sec	Prolonged fall Depth
6	120	130	130	0	30	3	0	0
7	200	154	17	0	34	3	2	0
11	150	153	0			2	50	0
12	120	165	0			2	10	0

approximately 25 cc of crude renin made according to the method of Grossman.[2] In earlier experiments saline extracts of kidney were used with similar results. The blood for transfusion was drawn at an arbitrary point where the recorded pressure curve regained the baseline. It was thought that the antipressor effect would probably be most prominent at this time. This needs further investigation.

Four experiments were carried out using normal donors and recipients. The donor of No. 11 received renin, the others did not (Table II).

No. 6 exemplifies a precipitous fall to a shock level and recovery during transfusion. No prolonged falls occurred. The blood pressures of unoperated dogs were often elevated in comparison with their preanesthesia levels. On the contrary, dogs with clamped renal arteries showed moderate depressions of pressure under anesthesia. The explanation is obscure.

Table III shows the effects of transfusing hypertensive dogs with blood of hypertensive donors. The donors had pressures of over 200 mm of mercury with and without anesthesia.

Immediately upon stopping the transfusion the pressure in No. 14 regained its initial hypertensive level.

A tendency appeared for the pressure to fall in direct proportion to the initial height. However, the limited number of observations is insufficient to establish the tendency as a rule.

Discussion. Houssay and Fasciolo[3] raised the blood pressure in

TABLE III.
Hypertensive Donor and Hypertensive Recipient.

No.	Quantity blood (cc)	Initial B.P. (mm Hg)	Transfusion fall Depth	Transfusion fall Duration min	Transfusion fall Duration sec	Duration transfusion min	Duration transfusion sec	Prolonged fall Depth
1	200	194	0			5	40	0
14	200	194	56	4	26	4	30	0

---

[2] Grossman, E. B., PROC. SOC. EXP. BIOL. AND MED., 1938, **39**, 40.

[3] Houssay, B. A., and Fasciolo, J. C., *Rev. soc. argent. biol.*, 1937, **13**, 284.

nephrectomized dogs by implanting ischemic kidneys into their necks. Recently, Solandt, Nassim and Cowan[4] demonstrated a rise in pressure of normal, nephrectomized dogs by cross-transfusion with hypertensive ones. This suggests that a pressor substance is produced by the kidney and is mediated through the blood stream. The experiments described above attempt to demonstrate the presence of an antipressor substance in the blood of dogs with intact kidneys and its absence in those with clamped renal arteries and hypertension. The antipressor effect is demonstrable only in hypertensive animals. Since the beginning of this work, Harrison, Grollman and Williams[5] and Page, Helmer, Kohlsdaedt, Fouts, Kempf and Corcoran[6] reported a latent and prolonged reduction of hypertension with material chemically extracted from kidneys. Although the crude materials used by them contain impurities, their work plus the demonstration of the absence of an antipressor effect in blood of dogs with partially occluded renal arteries given here indicate that the kidneys produce an antipressor substance. These experiments show that the antipressor effect is mediated through the blood stream. There appears to be an equilibrium in the circulation of normal dogs between a substance that tends to elevate the blood pressure and one that acts to prevent its elevation.

Transfusions between dogs that cross-match satisfactorily may cause deep falls of short duration that are clearly distinct from the physiological response of hypertensive dogs to the addition of nonhypertensive blood.

Conclusions. Blood of dogs with intact renal circulations reduces the blood pressure of those made hypertensive by partially occluding the renal arteries. Blood from hypertensive dogs gives no such effect. Blood of normal dogs contains an antipressor substance that is absent in hypertensive dogs. Normal blood pressures are not affected by it.

---

[4] Solandt, D. Y., Nassin, Reginald, and Cowan, C. R., *Lancet*, 1940, **1**, 873.

[5] Harrison, T. R., Grollman, Arthur, and Williams, J. R., Jr., *Am. J. Physiol.*, 1940, **128**, 716.

[6] Page, I. H., Helmer, O. M., Kohlsdaeht, K. G., Fouts, P. J., Kempf, G. F., and Corcoran, A. C., PROC. SOC. EXP. BIOL. AND MED., 1940, **43**, 722.

11624

A Right-Atrium Preparation for Studying Pace-Maker Activity and Amplitude of Contraction.

C. R. Spealman. (Introduced by R. J. Main.)

From the Department of Physiology and Pharmacology, Medical College of Virginia, Richmond.

A method for perfusing the isolated right-atrium of the guinea pig heart is illustrated in the schematic diagram. The first step in making such a preparation is to insert a cannula connected to a reservoir of Locke's solution into the inferior vena cava. A second cannula is then inserted into the superior vena cava. A ligature is now tied around the right-atrium at its junction with the right ventricle. In performing this third step, I have found it convenient to pass forceps caudally and to the left between the superior vena cava and the large arteries, through the left atrial tissue near its caudal junction with the right atrium. A ligature is then drawn through and tied at the right atrio-ventricular junction. The greater part of the two ventricles and the left atrium is excised, and the preparation is dissected free from the supporting tissue.

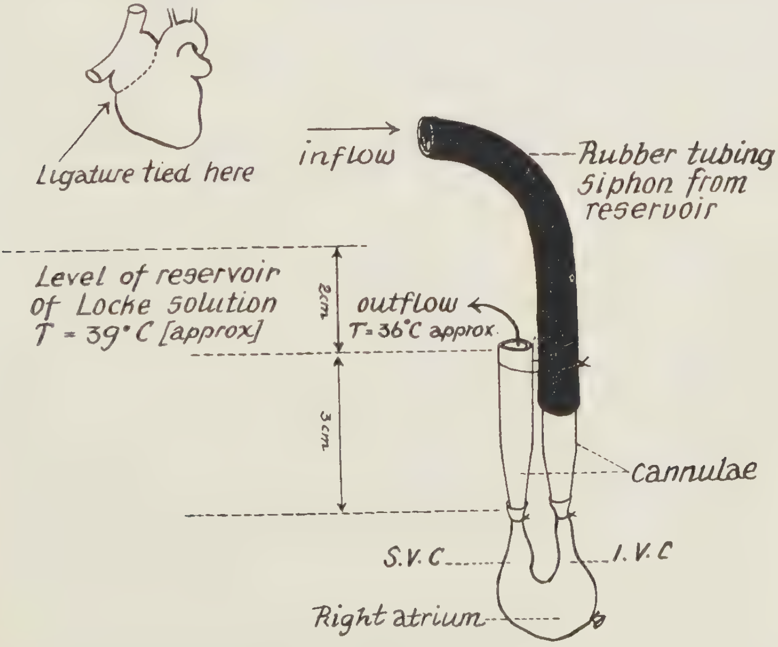

Ligature tied here
inflow
Rubber tubing siphon from reservoir
Level of reservoir of Locke solution T = 39° C [approx]
2cm
outflow
T=36°C approx.
3cm
cannulae
S.V.C
I.V.C
Right atrium

Fig. 1.

A schematic diagram of the atrium preparation. The inset in the upper left corner shows where the ligature is tied.

At this stage the preparation resembles a tube with a cannula in each end. The 2 cannulae are now loosely tied together. The preparation is clamped in a vertical position to a ring stand, and connected to a light lever by means of a thread. An end of the ligature about the atrio-ventricular junction is a convenient place to attach the thread to the atrium. In the resulting preparation, oxygenated Locke's solution at 36 to 37°C flows through one cannula and out the other. The various pressure levels are approximately those shown in the diagram; the chief aims in adjusting these levels are to have the atrium moderately distended and to have sufficient Locke's solution flowing through the preparation. (I have used about 10 cc per minute).

The preparation will continue to beat with a rather constant rate and amplitude for several hours. It responds to changes in the ionic composition of the Locke's solution and to epinephrine in the expected manner. Other substances have not been tried. The advantages of this preparation are its simplicity and durability.

11625

Effect of Diet and Cinchophen on Production of Experimental Gastric Ulcers in Chicks.

GARNETT CHENEY.* (Introduced by W. Dock.)

From the Department of Medicine, Stanford School of Medicine, San Francisco, California.

The production of superficial gastric ulcers in young chicks by a diet deficient in greens, whole grains, and certain fats is a relatively simple procedure. The lesions which develop are never in the proventiculous or acid-secreting area of the stomach, but are always in the muscular part and are best described as gizzard erosions. A number of factors which contribute to their development, prevention, and care have been described by Dam,[1, 2] Almquist and his coworkers,[3-6]

---

* Supported in part by a grant from Eli Lilly and Company.

[1] Dam, H., *Biochem. J.*, 1935, **29**, 1273.

[2] Dam, H., and Schonheyder, F., *Biochem. J.*, 1936, **30**, 897.

[3] Almquist, H. J., and Stokstad, E. L. R., *Nature*, 1935, **135**, 652.

[4] Almquist, H. J., and Stokstad, E. L. R., *J. Nutrition*, 1937, **13**, 339.

[5] Almquist, H. J., *J. Nutrition*, 1937, **14**, 241.

[6] Almquist, H. J., and Mecchi, E., *J. Biol. Chem.*, 1938, **126**, 407.

Cheney,[7, 8] and Esselen.[9] The lesions develop in approximately 50 to 100% of all chicks, the incidence depending upon the severity of the diet and the age of the bird. Chicks raised from hens on a deficient diet show a higher incidence of severe lesions.[10] These lesions occur in a stomach which secretes highly acid juice[8, 11] rich in pepsin[12] and they are usually located where this acid juice first strikes the lining of the muscular non-acid producing portion of the stomach or gizzard.

Histologically the lesions involve the chitinous gizzard lining of the lumen and the underlying mucosa only, and tissue necrosis does not occur. In the examination of over 2000 birds, the writer has never seen any penetrating or necrotizing ulceration from dietary deficiency alone. The dietary substance, an absence of which causes these erosions, has been termed the anti-gizzard erosion factor and was originally thought to be identical with vitamin K. Although it is closely associated with this vitamin, it is a separate substance as shown by Almquist.[4]

During the last 5 years the writer has carried out a number of experiments to try to produce lesions which were more than superficial and which would present the characteristics of true peptic ulcers as seen in the human stomach. These studies were fruitless until the last 6 months when cinchophen was fed to the chicks in their diet. This drug was chosen because of its well known capacity to produce gastric ulcers in dogs[13, 14] and cats.[15] The results of these cinchophen feeding experiments have been eminently successful not only in the production of penetrating and perforatory gastric ulcers typical of human peptic ulcers histologically, but also in demonstrating that a protective factor in the diet can nullify or modify ulcer production by cinchophen.

Some 250 chicks have been given diets containing cinchophen and a like number of controls have been observed. Examples of these experimental studies are shown in Table I. The basal diet (B, D in table) used is the same as that previously described[8] and is deficient in the anti-gizzard erosion factor. Twenty-five percent and 10% sup-

---

[7] Cheney, Garnett, *Arch. Int. Med.*, 1937, **60**, 705.

[8] Cheney, Garnett, *Am. J. Digest. Dis.*, 1938, **5**, 104.

[9] Esselen, W. B., *Poultry Science*, 1939, **18**, 201.

[10] Almquist, H. J., personal communication.

[11] Friedman, M. H. F., personal communication.

[12] Cheney, Garnett, unpublished work.

[13] Wagoner, F. H., and Churchill, T. P., *Arch. Path.*, 1932, **14**, 860.

[14] Stalker, L. K., Bollman, J. L., and Mann, F. C., *Arch. Surg.*, 1937, **35**, 290.

[15] Schwartz, S. O., and Simonds, J. P., PROC. SOC. EXP. BIOL. AND MED., 1935, **32**, 1133.

TABLE I.

Effect of Diet, Cinchophen Feeding, and Certain Protective Supplements Added to the Diet, on Grade of Ulceration in Stomachs of Chicks.

Series No.	Diet	No. of chicks in experiment		Cinchophen		Protective supplement	Age at autopsy days	Avg wt at autopsy g	Grade of ulceration
		Beg.	End	% of diet	Days fed				
S-1, 1 and 2	R.F.S.M.*	12	10	—	—	—	28	104.5	0.20
S-1, 4	,,	12	6	1.5	14	—	28	64.0	2.67
S-1, 3 and 5	,,	24	17	1	14	—	28	66.1	0.84
S-1, 7	,, + 25% alfalfa	12	0	1	14	—	28	—	—
S-5, 7	B.D.†	8	8	—	—	—	21	152.0	0.75
S-5, 6	,,	10	5	2	14	—	20	55.6	1.80
S-2, 4	,,	12	8	1.5	14	—	29	106.0	2.90
S-2, 6	,,	8	8	1.5	14	Fresh milk	28	131.6	1.63
S-3, 5	,,	12	8	1	10	—	21	98.3	2.20
S-3, 8	,,	12	9	1	10	Fresh milk + 1% M.G.‡	20	110.4	0.89
S-2, 9	,, + 25% alfalfa	6	6	—	—	—	28	102.5	0.17
S-5, 8	,, ,, ,,	10	1	2	14	—	19	—	—
S-2, 8	,, ,, ,,	9	8	1.5	14	—	29	94.75	4.0
S-3, 6	,, ,, ,,	12	12	1	10	—	21	96.7	2.67
S-3, 7	,, ,, ,,	7	7	0.5	10	—	21	108.7	2.0
S-4, 1	,, + 10% ,,	7	7	—	—	—	21	173.14	0.07
S-4, 3	,, ,, ,,	10	8	1.5	11	—	22	78.25	4.0
S-4, 8	,, ,, ,,	10	6	1.5	11	5% Cerophyl	22	96.0	1.75
S-4, 6	,, ,, ,,	10	8	1.5	12	1% ,,	23	80.75	2.6
S-4, 7	,, ,, ,,	10	10	1.5	8	5% MG‡	23	110.6	2.35
S-4, 5	,, ,, ,,	10	8	1.5	11	1% ,,	22	89.5	2.7
S-4, 4	,, ,, ,,	10	7	1.5	10	0.5 cc bile daily	21	86.6	3.2
S-3, 4	,, ,, ,,	10	10	1.0	10	—	20	154.5	2.55

*R.F.S.M.—Equal parts rice flour and skimmed milk powder.

†B.D.—Basal diet (see text).

‡M.G.—Dried canned mixed greens (Heinz).

plements of dry alfalfa were added to it to give bulk. The larger percent of alfalfa ordinarily gives moderate protection against erosions, but interferes with the nutritive value of the diet, while the 10% level offers only slight protection. A diet of equal parts of rice flour and dried skimmed milk powder was originally used (R. F. S. M. in table) which contained the anti-gizzard erosion factor, but it did not prove satisfactory as the control birds failed to gain weight satisfactorily and ultimately developed "crazy chick disease" reported as vitamin E deficiency.[16]

Cinchophen (Merck) was added to the diet at levels from one-half to 2%. Dietary supplements containing the anti-gizzard erosion factor were added at levels of one and 5% in the form of dried canned mixed greens (asparagus, lettuce, and kale)† and cerophyl (dried grass stalks)‡ and as fresh whole pasteurized milk substituted for drinking water, as these substances have previously been shown to be excellent sources of the factor.[12] Bile was added in one experiment in accordance with Almquist's findings that it is highly protective against gizzard erosions.[6]

The birds were raised as already described;[8] and the various special diets were started from the third to the fourteenth day after hatching.§ Cinchophen feeding was not started until 5 to 7 days later, except in the fourth series of birds (S-4 in the table) when it was started simultaneously with the supplemental diet. Six to 12 chicks were utilized for each experiment and the survivors were autopsied during the fourth week of life as a rule. Post-mortem examination was carried out on birds dying while under observation and on those which were well preserved. The average weight of the chicks at the time of autopsy is recorded in the table as an indication of their state of nutrition at the time of death.

As an indication of the severity of the lesions which were produced, they are scored in the table on a basis of depth as follows: Grade 1, hemorrhage in the mucosa but without any break in the overlying chitinous layer. This is the earliest change which occurs. Grade 2, an erosion of the superficial gastric lining and mucosa but without evidence of any reaction in the underlying structures. Grade 3, similar to Grade 2 but the lesion has a punched-out appearance often with indurated margins and with a flat base on the submucosa or underlying muscle layer. Grade 4, definite penetrating ulcers with muscular tissue invaded. Grade 5, a perforated ulcer with localized or generalized peritonitis. Grades 1 and 2 are found in dietary deficiency alone; grades 4 and 5 only when cinchophen has been added. Grade 3 is rare in dietary deficiency alone, but very common with cinchophen feeding. Multiple lesions of varying depth were not infrequently encountered. They were graded according to the deepest lesion.

---

[16] Pappenheimer, H. M., Goettsch, Marianne, and Jungherr, Erwin, Bulletin 229, Connecticut State College, Storrs, Conn.

† Supplied through the courtesy of Heinz and Company.

‡ Supplied through the courtesy of the Cerophyl Company.

§ All birds were hatched and supplied through the courtesy of Poehlman's Hatchery in Petaluma, California.

Experimental Results. It is evident from the data presented in the table that the diets devoid of roughage were not very satisfactory as severe grades of ulceration were unusual. Chicks fed on this type of mash diet often do not gain well[17] and the yellow liquid "mustard water" droppings which they passed indicated that the cinchophen might have been eliminated too rapidly for complete absorption to take place. The diet with 25% roughage proved unsatisfactory because the general nutrition of the birds was interfered with. The group of chicks receiving the basal diet plus 10% roughage not only gained weight satisfactorily, but showed a high percent of lesions of the peptic ulcer type when cinchophen was added.

It is also evident that the level of 2% cinchophen in the diet was too toxic for satisfactory experiments, as many of these birds died within 24 to 48 hours with loss of appetite and profound weakness and prostration. Very few survived the period of the experiment. A number of birds in Groups S-5, 6, and S-5, 8 apparently died hepatic deaths. At autopsy the livers were tan to yellow ochre in color and histologically showed marked infiltrations of fat. No definite evidence of hepatitis was found. The only other group of birds which may have suffered liver damage was S-2, 3 which received 1.5% cinchophen and 2% bile salts.¶ Five of these birds were satisfactory for autopsy study and showed small tan livers, huge distended gallbladders and ascites. Histologically the livers showed no evidence of hepatitis.

In general the evidence of severe gastric lesions diminished in direct proportion to the amount of cinchophen fed and when the drug was at 0.5% level, the lesions were only slightly more pronounced than those obtained on a deficient diet alone. With the higher concentrations of cinchophen, the initial lesion was evident in the first 24 to 48 hours but with the lower levels not until the third or fourth day of feeding. Perforations occurred as early as the fifth day but more often between the seventh and fourteenth days.

---

[17] Hart, E. B., Halpin, J. G., and Steenbock, H., *J. Biol. Chem.*, 1920, **43**, 421.

¶ This group has not been included in the table as only two of seven birds survived the experiment. They ate very little of the mash and failed to gain weight.

When protective supplementary substances containing the anti-gizzard erosion factor were added to the diet a definite diminution in the severity of the lesions was noted even when these substances were fed at only a one percent level. This was particularly noticeable in Group S-3, 8 when whole milk was also added to the diet. The very low grade of ulceration obtained in this group of chicks receiving one percent cinchophen should be compared with the control group S-3, 7 which received 0.5% cinchophen, as the chicks receiving milk consumed only a little over one-half as much feed as this group and consequently proportionately less cinchophen. The results of protective feeding in Group S-4, 8 are particularly worthy of note. The grade of lesions for this whole group including the 5 birds which succumbed from perforation before the experiment was completed was 2.95, but for the 5 survivors was only 1.75. As this group of birds did not receive the protective substance before cinchophen feeding was started, the results indicate that protection was beginning to develop after the first 5 to 7 days and that the good end results were due to healing of previously formed lesions.

The failure of fresh bile to show a beneficial effect on the gastric lesion is difficult to explain. Further studies of a similar nature must be carried out. Possibly hepatic function is impaired by the cinchophen so that the bile cannot be satisfactorily utilized. Perhaps bile simply facilitates the absorption of the anti-gizzard erosion factor and this function is impaired by impaired liver function. In this connection it is of interest to note that dogs with biliary fistula and complete loss of bile develop a high incidence of gastric ulceration even when maintained on a normal diet.

Conclusions. 1. Feeding cinchophen to chicks produces gizzard lesions with the characteristics of peptic ulcers. 2. These gastric ulcers vary in severity with the amount of cinchophen fed. 3. The early lesions are similar to those produced by a dietary deficiency alone. 4. The lesions are less severe in chicks fed a dietary supplement containing the anti-gizzard erosion factor.

11626

Variations in Alkali Reserve of Plasma and Excretion of Total Titratable Urinary Acid in Hyperthyroidism.

WILLARD BARTLETT, JR. (Introduced by M. Somogyi.)

From the Laboratory of the Jewish Hospital and the Department of Surgery, St. Louis University School of Medicine.

The literature on the alkali reserve of the plasma in hyperthyroidism is small and is inconclusive, particularly in view of the scarcity of serial studies[1-8] and the inclusion of complicated cases. Accordingly, we made serial observations on 15 hospitalized patients between 1933 and 1935 and the pertinent findings are given in Table I. Similar observations on 3 patients in a more recent series appear in Table IV. None of these patients had any complicating metabolic disease and one had cardiac decompensation; the occurrence of auricular fibrillation in 3 patients in Table I is noted. All estimations were made in the basal state: blood was taken from the unobstructed vein, usually under oil, into tubes containing neutral potassium oxalate, centrifuged immediately and the CO_2 combining power of the separated plasma was determined, ordinarily at once, by the method of Van Slyke and Cullen.[9] Rarely, the separated plasma was kept in the ice box, but the determination was made within one hour of vena puncture. Postoperative observations were made on the fifth to seventh day. It seemed suggestive that, of the 18 patients, 8 showed a rise of from 3 to 14 vol. % with improvement of the thyroid status and that in the more severe stages of hyperthyroidism values for CO_2 combining power tended toward the lower limits of the normal range. There was, clearly, no direct correlation between the level of basal metabolism alone and the value for CO_2 combining power of the plasma.

In view of these findings further investigation of the acid-base regulatory mechanisms were made by serial determinations of the total titratable acid of the urine.[10] Another series of patients was

---

[1] Davies, H. W., Meakins, J., and Sands, J., *Heart*, 1924, **11**, 299.

[2] Walinski, F., and Herzfeld, E., *Muenchen med. Wchnschr.*, 1926, **73**, 2153.

[3] Damble, K., and Reuter, A., *f. Klin. Med.*, 1933, **125**, 690.

[4] Coelko, E., *Endokrin.*, 1932, **10**, 74.

[5] Altenburger, E., and Boger, A., *Klin. Wchnschr.*, 1933, **12**, 1983.

[6] Skold, E., *Arch. f. Klin. Chir.*, 1928, **151**, 600.

[7] Zondek, H., *Deutsch. med. Wchnschr.*, 1929, **55**, 345.

[8] Koenig, W., *Arch. Klin. Chir.*, 1931, **164**, 213.

[9] Van Slyke, D., and Cullen, G. E., *J. Biol. Chem.*, 1917, **30**, 289.

[10] Folin, O., *Am. J. Physiol.*, 1903, **9**, 25.

hospitalized and kept until operation on diets which were constant in the quantities of basic and acid-forming components and of constant caloric intake. The diets necessarily varied somewhat, one from another, to suit the food preferences and eating habits of the individual patients. A period of 2 or 3 days was allowed to elapse before collection of 24-hour urines in stoppered bottles containing 2 cc of toluene was begun. (Bottles and collecting vessels were sterile in Case 9 in whom organic acid and ammonia nitrogen were also determined.) All estimations were made in duplicate. The diet was resumed on the third or fourth postoperative day.

The data on 9 cases in which this constant regime could be maintained appear in Table II. Characteristic excerpts for consecutive days at 3 intervals during hospitalization, with the mean acidity and the range during such period are presented. (The complete protocol of Case 9 covers 8 weeks.) A consistently close relationship exists between the course of the individual case and the quantity of acid excreted. Where prompt relief of hyperthyroidism occurs shortly after operation, a fall to one-half or one-fourth of the original level is seen; indeed, in Case 3, urine alkaline to phenolphthalein was obtained on two occasions postoperatively. Where the clinical im-

TABLE I.
Basal Metabolic Rate and Carbon Dioxide-Combining Power of Plasma Before and After Thyroidectomy.

	Unstab.		Pre-op.		Post-op.	
Ex. G.	BMR	CO_2 combining power of plasma volume % CO_2	BMR	CO_2 combining power of plasma volume % CO_2	BMR	CO_2 combining power of plasma volume % CO_2
1. 30, M	+91†	50	+32†	60	+ 4	64 d
2. 50, F	+75?	56			+10	56
3. 22, M	+70?	61	+16	60	+11	59 d
4. 39, F	+40	55	+37	53*	+ 7	53
5. 42, F	+80?†	51	+42	52*	+24	59
6. 44, F			+34†	54	+16†	55 d
7. 23, F	+26	58	+14	62	— 4	65 d
8. 29, M	+64	60	+23	61	+19	60 d
9. 37, F			+16	57	+ 5	60 d
Tox. Ad.						
10. 59, F	+32	72	+14	68	+15?	74
11. 38, F			+22	61	—15	57
12. 48, F			+25	62	— 9	67
13. 36, F	+45	57			+ 2	67
14. 27, F			+ 3	61	0	59
15. 52, F			+31	59	+16	58

*Vena puncture difficult.
†Auricular fibrillation.
d. 2-stage thyroidectomy.

TABLE II.
Total Titratable Acid of Urine in Hyperthyroidism.

Case	Unstabilized (admitted)			Pre-op.			Post-op. (discharged)		
	Days	Acid cc 0.1N	BMR	Days	Acid	BMR	Days	Acid	BMR
1. 52, F	4	270 (334-204)	+38	2	93 (136- 50)	+20	3	85 (122- 62)	
2. 44, F				5	237 (299-153)	+18	3	251 (291-209)	+19
3. 48, F	1	180	+38	4	64 (84- 46)	+17	4	18 (51- 0)	+10
4. 29, F	7	316 (422-257)	+41	3	247 (263-233)	+15	3	128 (136-111)	+ 4
5. 56, F	7	276 (326-207)	+29	3	193 (273-153)	+21			
				3	155 (228-111)	+11	4	126 (163- 68)	+ 4
6. 32, F						+38	5	195 (249-142)	+12
							3	92 (90- 94)	
7. 17, F	6	382 (455-294)	+38						
							5	136 (204- 73)	+ 5
8. 60, F	3	314 (348-262)	+36	6	197 (252-100)	+31			
							9 { 5	327 (429-185)	
							4	220 (238-177)	+19
9. 19, F	5	357 (388-315)	+50	10	135 (193- 86)	+24			
						+16	6	96 (147- 65)	—13

provement over the preoperative status is slight in the early postoperative phase (Cases 2, 8), little or no change in total acid excretion occurs as expressed in averages. In Cases 5, 6, 7, 8, 9 two-stage thyroidectomy was done; preoperative observations on Cases 8 and 9 precede first lobectomy in both instances. All postoperative observations in two-stage cases (except Case 6) are subsequent to the second lobectomy, hence they appear on the line below that on which admission observations are recorded. Physical activity in all cases was limited to getting from bed to a chair and to the bathroom.

Table III presents further excerpts from the protocol of Case 9 and it is seen that in the brief phase in which she is temporarily getting worse (on 1/27 and 1/28) her organic acid excretion[11] and ammonia nitrogen[12] rise, along with the total titratable acid, and that the pH of the urine falls. These associations were quite constant throughout the prolonged period of observation. On the contrary, the opposite trends appear in the findings on her last 4 days before discharge following her second lobectomy at a time when she was improving rapidly.

During this study of acid excretion, we resumed the estimation of

TABLE III.
Constituents of 24-hour Urine Before and After Thyroidectomy. Extracts of Protocol of Case 9.

	Case 9.								
	Pre-op					Post-op.			
Date	1/25	1/26	1/27	1/28		3/6	3/7	3/8	3/9
Vol.	2100	1500	2100	2650		3100	2750	2350	2740
T. acid	142	102	172	190	Op.	99	99	65	65
O. ”	26.8	14.4	20.1	33.9	2/7	35.9	26.4	26.3	21.9
NH_3 N	.239	.211	.281	.439	and	.558	.481	.439	.446
pH	6.0	6.0	5.8	5.8	2/29	6.5	6.5		7.0
BMR				+32					—13

Total acid, cc 0.1N per 24 hr.
Organic acid, milli eq. per 24 hr.
Ammonia N, g per 24 hr.

TABLE IV.
CO_2 Combining Power of Plasma and Urinary Acid.

	Unstabilized			Pre-op.			Discharge		
Case	Acid	CO_2*	BMR	Acid	CO_2*	BMR	Acid	CO_2*	BMR
3.	180	59	+38	64	60	+17	18	66	+10
4.	316	56	+41	247		+15	128	60	+ 4
8.	314	60	+36	197	58	+31	220	60	+19

*CO_2 combining power of plasma, vols %.

---

[11] Van Slyke, D., and Palmer, W., *J. Biol. Chem.*, 1920, **41**, 567.

[12] Folin, O., and Bell, R., *J. Biol. Chem.*, 1917, **29**, 329.

CO_2 combining power of the plasma in 3 cases and these results appear in Table IV. In Cases 3 and 4, in which prompt improvement of thyroid status occurred, a definite rise in CO_2 combining power accompanied the fall in excretion of total acid; in Case 8, clinical improvement did not occur so soon after operation and no rise in alkali reserve or fall in urinary acid excretion is seen. Further studies are needed to determine the existence of a correlation here.

The tendency of severely thyrotoxic patients to show impaired liver function is firmly established, yet no correlation can be shown between the severity of hyperthyroidism and the extent of functional hepatic deficiency. Somogyi[13] has recently demonstrated important accumulations of ketone bodies in the blood of hyperthyroid patients and while we have not identified the organic acids, the excretion of which is so largely increased in Case 9 (rising above 50 milli-equivalents at one time), we should expect them to be beta-hydroxybutyric and aceto-acetic acids. We assume the increased ammonia nitrogen excretion in this case to be an indication of stress on base economy of the body during the exacerbations of hyperthyroidism.

Summary. Serial observations on 18 patients reveal a rise of carbon dioxide-combining power of the plasma in 8 cases as their clinical status improves with treatment and operation. All values fall within the normal range. The excretion of total titratable acid of the urine in 9 patients on constant diets is reported. In the patients in whom decided improvement occurs shortly after operation, the preoperative amounts are from 2 to 4 times as large as those excreted postoperatively. Excretion of organic acids and ammonia nitrogen were also followed for nearly a month in one patient and in exacerbations of hyperthyroidism her excretion of these substances, as well as of total acid, is seen to rise; urinary pH values fall during exacerbations. In 2 patients who improved promptly following thyroidectomy, fall in total acid excretion is accompanied by a rise of alkali reserve; in a third patient who showed little early improvement and only a slight change of total acid excretion, there was no change in carbon dioxide-combining power of the plasma.

These findings are consistent with the existence of a compensated acidosis in hyperthyroidism.

---

[13] Somogyi, M., personal communication.

11627

Increased Susceptibility to Chloroform Poisoning Produced in the Albino Rat by Injection of Crystalline Thyroxin.

Monroe A. McIver.

From the Surgical Service of the Mary Imogene Bassett Hospital, Cooperstown, N. Y.

There is a considerable volume of literature dealing with the deleterious effects upon the liver of abnormal amounts of thyroid hormone. These changes in the liver have been found in laboratory animals following ingestion of thyroid preparations,[1, 2] and have been reported from clinical and pathological studies on human beings.[3-9] Briefly, they consist in different types of degenerative processes in the liver, detected by histological examination of post-mortem material, and in diminished liver function as shown by various tests.

There is much difference of opinion as to the importance of liver changes occurring in hyperthyroidism. Many believe that they may be of considerable significance in the clinical picture, particularly in connection with the thyroid crises and postoperative "storms." Others hold that they are not of basic significance: Means,[10] for example, believes that they are not due to direct injury by the thyroid hormone. Whatever may be the correct interpretation of the observed phenomena, however, it is probably well established that degenerative changes in the liver, and diminished liver function, are often associated with hyperthyroidism; and that jaundice not infrequently occurs in connection with a thyroid crisis.

The following experiments were undertaken primarily to discover whether the picture produced by liver injury in the presence of an increased amount of thyroid hormone in the circulation is different

---

[1] Farrant, R., *Brit. M. J.*, 1913, 1363.

[2] Hashimoto, H., *Endocrinology*, 1921, **5**, 579.

[3] Cameron, G. R., and Karunaratne, W. A. E., *J. Path. and Bact.*, 1935, **41**, 267.

[4] Weller, C. V., *Ann. Int. Med.*, 1933, **7**, 543.

[5] Goodpasture, E. W., *J. Am. Med. Assn.*, 1921, **76**, 1545.

[6] Boyce, F. H., and McFetridge, E. M., *Arch. Surg.*, 1938, **37**, 401.

[7] Bartels, E. C., and Perkin, H. J., *New England J. Med.*, 1937, **216**, 1051.

[8] Maddock, W. G., Pedersen, S., and Coller, F. A., *J. Am. Med. Assn.*, 1937, **109**, 2130.

[9] Beaver, D. C., and Pemberton, J. deJ., *Ann. Int. Med.*, 1933, **7**, 687.

[10] Means, J. H., *The Thyroid and Its Diseases*, pub. Phila., J. B. Lippincott, 1937, p. 292.

from that produced by liver injury unassociated with hyperthyroidism.

Method. The experimental animal was the albino rat; liver injury was produced by injection of chloroform. This animal and drug were selected because of the extensive data that have been accumulated on the effects of chloroform on rats. The rats were in healthy, active condition. They were weighed every 3 days, and also within 24 hours before the injection of chloroform. In most instances the body temperature was taken by rectum once or more daily. The animals were kept on a standard diet (Purina Dog Chow).

Crystalline thyroxin (Squibb's) was administered daily, by subcutaneous injection, over a period varying from 7 to 20 days,* in doses varying from .1 to .2 mg, the crystals being dissolved in an alkaline solution and the total volume for each dose made up to ½ cc with sterile distilled water.

At the end of the period of thyroxin administration, the animals were injected subcutaneously with varying doses of chloroform (Squibb's) in mineral oil. The doses of chloroform were based on the findings of Moise and Smith,[11] that the majority of their animals on standard diet receiving 1.5 cc of chloroform per kg of rat survived. Opie and Alford[12] have reported the survival of animals on standard diet with as high a concentration of chloroform as 2.0 cc per kg of rat. In the present experiments, the chloroform varied in amount from 1.5 to .4 cc per kg of rat, and was diluted with sterile mineral oil so that the total volume per dose was .5 cc.

All the animals that died were autopsied as soon as possible. Certain animals were sacrificed for histological study, as indicated in Table I.

Results. The animals, during the period in which they were receiving thyroxin injections, showed few if any clinical symptoms—although they were perhaps more active and lively than the control animals. In general, their weight was well maintained or, in a few instances, increased, and their hair remained in good condition.

The results of the chloroform injections are shown in Tables I and II. Table I includes rats receiving from 1.5 to .8 cc of chloroform per kg of body weight. The survival period of these animals varied from 12 to 36½ hours, with the exception of one rat who made a complete recovery. The survival period for the control animals who

---

* A few animals received desiccated thyroid tablets, but they were too few in number to include. The results, however, would seem to be comparable with the crystalline thyroxin.

[11] Moise, T. S., and Smith, A. H., *J. Exp. Med.*, 1924, **40**, 13.

[12] Opie, E. L., and Alford, L. B., *J. Am. Med. Assn.*, 1914, **62**, 895.

TABLE I.

Tables I and II, Survival Period of Rats Following the Injection of Chloroform, with and without Previous Administration of Thyroxin.

Rat No.	Initial wt, g	Administration of crystalline thyroxin		Wt at time chloroform injected, g	Dose of chloroform per kilo of body wt, cc	Survival period	Remarks
		Total amt mg	Duration days				
4	215	1.3	10	190	1.5	12 hr	
17	210	2.0	16	214	.8	16½ ”	
7	209	.7	7	167	1.5	18½ ”	
30	204	2.4	16	233	.8	18½ ”	
31	228	2.4	16	247	1.5	23 ”	
22	183	2.0	13	228	.8	24 ”	
28	201	3.2	20	233	1.5	35 ”	Rec'd .2 mg cryst. thyrox. in 1 dose, after chloroform.
21	185	2.0	13	232	.8	36½ ”	
27	214	2.4	16	233	.8	Recovered	
		Control					
48	205			203	1.5	4 days	
56	166			185	1.5	5 ”	
46	173			191	.8	5 ”	
52	177			191	.8	5 ”	
32	138			158	.8	5 ”	
42	157			191	1.5	6 ”	
50	188			196	1.5	Recovered	
47	179			191	.8	”	
43	138			147	.8	”	
39	166			204	.8	”	
37	170			193	.8	”	
33	119			137	.8	”	
8	182			156	1.5	Sacrificed at 48 hr	No symptoms of chloroform poisoning.
11	218			210	1.5	Sacrificed at 48 hr	” ” ” ” ”

TABLE II.

Rat No.	Initial wt, g	Administration of crystalline thyroxin		Wt at time chloroform injected g	Dose of chloroform per kilo of body wt cc	Survival period	Remarks
		Total amt mg	Duration days				
24	192	2.0	15	222	.4	12½ hr	
26	162	2.4	16	179	.4	13½ ,,	
14	216	2.0	16	213	.4	17½ ,,	Temp. 104.6° on day of death.
29	153	2.4	16	147	.4	Recovered	Rec'd .6 mg cryst. thyrox. in 3 doses, after chloroform.
23	197	2.0	13	210	.4	,,	
12	210	.7	8	215	.4	,,	
		Control					
35	110			120	.4	,,	
34	128			141	.4	,,	
44	138			171	.4	,,	
54	152			190	.4	,,	
55	155			181	.4	,,	
57	178			190	.4	,,	

received corresponding doses of chloroform without the preceding administration of thyroxin, varied in 6 instances from 4 to 6 days, while 6 rats recovered. In 2 other instances the rats were sacrificed at the end of 48 hours for histological study; they showed no symptoms of chloroform poisoning. Table II shows a group of 6 animals who, following administration of crystalline thyroxin, received .4 cc of chloroform per kg of body weight. Three of these animals survived for 12½, 13½ and 21 hours, respectively; the other 3 recovered. Of the 6 controls who received .4 cc of chloroform per kg of body weight, all recovered.

Histological Studies. On autopsy, the animals which had received thyroxin injections followed by chloroform showed extensive liver damage, the lesions consisting of varying degrees of central necrosis, usually extreme. The kidney lesions were not impressive, the changes consisting merely in cloudy swelling. In the majority of the cases, examination of the lungs showed marked engorgement of the vessels of the alveolar walls, and evidence of edema. In a few instances there was actual hemorrhage into the alveolae.

Control animals Nos. 48, 56, 46, 52, 32, and 42, died from 4 to 6 days after administration of chloroform. In some instances it was difficult to find evidence of damage to the liver; in other instances there was evidence of reparatory processes. It seemed unlikely that the liver pathology had been responsible for the death of any of these animals. The kidneys showed interesting lesions, the most outstanding being extensive damage to the convoluted tubules, with areas of calcification, the picture resembling that seen in bichloride of mercury poisoning.

Discussion. The above results would seem to indicate that rats on a standard diet who have been injected with crystalline thyroxin over a period of time show increased susceptibility to chloroform poisoning.† As has long been known,[12] the action of chloroform is profoundly influenced by diet—starvation, or diets high in fat, rendering the animals particularly susceptible to chloroform poisoning, while high carbohydrate and high protein diets afford considerable protection.[11, 15, 16] Since the administration of thyroid preparations affects the glycogen content of the liver, as shown by Coggeshall and Greene,[17] it might be supposed that this was a prime factor in the increased susceptibility to chloroform poisoning in hyperthyroidism. However, it is by no means certain that the explanation is so simple: and in a few preliminary experiments of the present study we were not able to protect the animals who were receiving thyroxin, from the effects of chloroform, by supplementing the diet with intraperitoneal injections of glucose.

---

† The fact that administration of thyroid preparations may influence the action of a drug was first demonstrated by the well-known experiments of Hunt[13] in which protection against the toxic effects of acetonitril was afforded to mice by the administration of the thyroid hormone. The only experiments which I have found on chloroform poisoning in the presence of hyperthyroidism were carried out by Davis and Whipple[14]: following the influence of Hunt's work they fed thyroid hormone to dogs prior to the administration of chloroform, but found that it was without effect.

[13] Hunt, R., *J. Biol. Chem.*, 1905, **1**, 33.

[14] Davis, N. C., and Whipple, G. H., *Arch. Int. Med.*, 1919, **23**, 612.

The mechanism whereby hyperthyroidism in rats increases their susceptibility to chloroform poisoning would seem to be worth further investigation, in the hope that it might throw some light on the relation between thyroid and liver, particularly as manifested in the thyroid crisis. It is interesting also to speculate as to the possible application of the present findings to the choice of anesthetic for operations upon patients with hyperthyroidism. Although few anesthetics have as deleterious an effect upon the liver as does chloroform, a number of them are to a certain degree harmful to this organ; and it may prove that their ill effects as well as those of chloroform are intensified in the hyperthyroid patient. This may be of particular significance in regard to tribromethanol (avertin) because of the chemical relationship of this drug to chloroform.

Conclusions. The susceptibility of the albino rat to chloroform poisoning is increased by the administration of crystalline thyroxin.

I wish to express my thanks to Dr. C. D. Ayer for his kindness in going over the pathological material with me.

---

[15] Goldschmidt, S., Vars, H. A., and Ravdin, I. S., *J. Clin. Inv.*, 1939, **18**, 277.

[16] Miller, L. L., and Whipple, G. H., *Am. J. M. Sc.*, 1940, **199**, 204.

[17] Coggeshall, H. C., and Greene, J. A., *Am. J. Physiol.*, 1933, **105**, 103.

11628

A Mercury Valve for Obtaining Alveolar Air Samples.

ROLLAND J. MAIN.

From the Department of Physiology, Medical College of Virginia, Richmond.

In obtaining alveolar air by the Lindhard method of securing the last portion of each expiration,[1] most investigators have collected the sample over aqueous solutions. In order to obtain the advantages of collecting it over mercury, the following valve was designed, employing a tuberculin syringe with a slot filed through the barrel, and enclosed in a glass shell, with an electromagnet to activate the plunger (Fig. 1). A nasal catheter (No. 8 Fr.) is passed through a nostril to the base of the uvula, and connected to the Haldane gas tube with Rehfuss tubing.

The gas tube is filled with mercury from the reservoir, and the clamp below is closed. A 10 cc syringe with a short piece of rubber tubing is connected to the short arm of the top cock of the Haldane gas tube, with the cock turned to open to the tubing. At the end of each of several expirations, a few cubic centimeters of air is rapidly drawn into the syringe. This flushes the dead space of the tubing and catheter, which approximates 1 cc. The cock is then turned to connect the mercury filled gas tube to the tubing.

At the end of each expiration (as can be noted by pasting a wisp of cotton to the nostril or with a chest pneumograph) the key is pressed down for 1 or 2 seconds. The mercury level in the gas tube promptly drops, drawing in several cubic centimeters of air. A minimum of rubber tubing should be used on the valve, to prevent lag and rebound, and the tubing used should be thick walled. If desirable electrical contacts on a pneumograph to activate the valve in place of the key could be used.

Results with this method during normal respiration, however, showed distinctly lower percentages of CO_2 than did the Haldane-Priestley method (at the end of expiration). This may be due to the following factors: (1) that a normal expiration may not completely wash out the dead space of the respiratory tract, (2) that the expiratory sample of the Haldane-Priestley technic is obtained slightly later in the respiratory cycle, than is the Lindhard sample, and (3) that the Lindhard sample shows the average CO_2 over a period of time, during which it may vary, whereas the Haldane-Priestley

---

[1] Lindhard, J., *J. Physiol.*, 1911, **42**, 337.

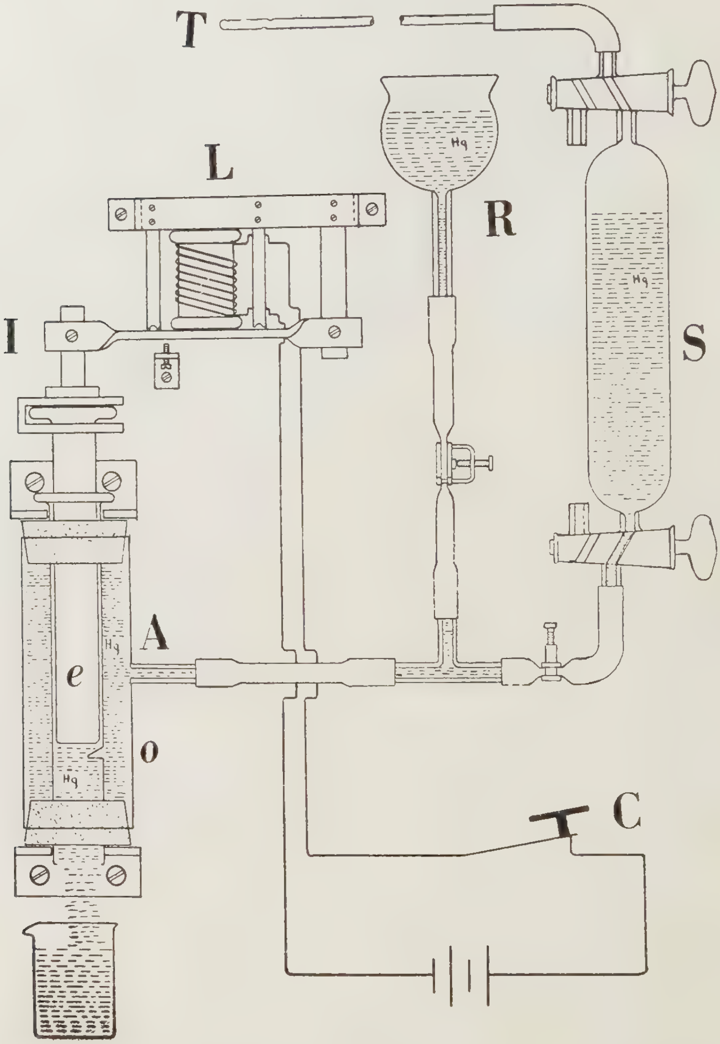


FIG. 1.

An electrically activated mercury valve for obtaining gas samples over mercury. Switch C is closed and the current of 2 dry cells activates the tripole electromagnet L, iron bar I is drawn upwards, pulling up plunger e of the tuberculin syringe. This syringe is inside the glass shell A with a side arm. Movement of plunger e upwards opens slot o filed through the barrel of the syringe, allowing the mercury to come in the side arm A, down the shell, through the slot into the barrel of the syringe, and down and out into the beaker below. This will lower the mercury level in gas tube S, thus sucking in air from nasal catheter T. Reservoir R is merely to refill gas tube S. When taking a sample, clamp under R is closed and clamp under S is open. Clamp below S tube is closed only when removing gas tube. The buret of the gas analysis apparatus could replace the tube S.

sample is an immediate index. In an attempt to obviate these first two objections, the subjects were made to breathe in the tempo set

TABLE I.

Subject	Lindhard % CO_2	Haldane-Priestley % CO_2
A	5.08	5.4
B	4.62	4.93
C	5.2	5.3
D	5.22	5.22

by a revolving electrically driven marker, so that they would breathe deeply at a rate of 6 times per minute. Haldane[2] has proved that voluntarily altering the rate of respiration does not affect the alveolar CO_2 percentage. However, the Lindhard samples were usually still slightly lower than the Haldane-Priestley samples as shown by Table I.

To obtain comparable results to the Haldane-Priestley method, it would probably be necessary to place the catheter further down the respiratory tract, as can be done with an anesthetized patient.

Summary. A mercury valve is described for obtaining samples of alveolar air over mercury at the end of expiration by a nasal catheter. The CO_2 values are lower than the Haldane-Priestley values, even when respiration is voluntarily slowed.

11629

Susceptibility of Spadefoot Toad and Tree Frog to Ouabain, Cymarin, and Coumingine Hydrochloride.

ALBERT P. BLAIR, CHESTER C. HARGREAVES AND K. K. CHEN.

From the Department of Zoology, Indiana University, Bloomington, and the Lilly Research Laboratories, Indianapolis.

Almost 85 years ago Vulpian[1] pointed out that the toad had a natural tolerance to digitalis. Subsequent investigators in confirmation with Vulpian's work reported that the toad was resistant to other cardiac drugs such as antiarin and strophanthin,[2, 3, 4] ouabain, cymarin, and coumingine hydrochloride.[5, 6] The African clawed toad *(Xenopus laevis)*, however, behaves more like the frog than the true toad *(Bufo)*.[4, 7] In invertebrates Evans[8] found that antiarin and gitalin were inactive on the snail's heart *(Helix pomatia)* although strophanthin produced its typical effect. Straub[9] obtained negative results with antiarin on the sea-hare's heart *(Aplysia)*. The grass snake *(Tropidonotus natrix)* is very tolerant to strophanthin as compared with the frog.[10] Among warm blooded animals, the rat has been shown[11, 12] to have a high tolerance to strophanthin and digitalis.

---

[2] Haldane, J. S., *Am. J. Physiol.*, 1915, **38**, 20.

[1] Vulpian, E. F. A., *Compt. rend. Soc. biol.*, 1856, **3**, 125.

[2] Fornara, D., *J. de thérap.*, 1877, **4**, 882.

[3] Heuser, O., *Arch. internat. de pharmacodyn. et de thérap.*, 1902, **10**, 483.

[4] Epstein, D., *J. Pharm. and Exp. Therap.*, 1931, **43**, 697.

[5] Chen, K. K., and Chen, A. L., *J. Pharm. and Exp. Therap.*, 1933, **47**, 295.

[6] Chen, K. K., Hargreaves, C. C., and Winchester, W. T., *J. Am. Pharm. A.*, 1938, **27**, 307.

The spadefoot toad, *Scaphiopus holbrookii holbrookii*, family *Scaphiopodidae*, has a pair of well-defined parotoid glands resembling in appearance those of the true toad. Phylogenetically, the spadefoot toad is generally considered more primitive than the true toad, family *Bufonidae*. The tree frog, *Hyla cinerea cinerea*, family *Hylidae*, on the other hand, is devoid of any structure suggestive of the parotoid glands, and in this respect it appears to be closer to the true frog, *Rana*.

The purpose of the present report is to show that, first, the venom from the spadefoot toad's glands contains no digitalis-like principles; and secondly, the spadefoot toad is less susceptible than the true frog to digitalis-like principles, such as ouabain, cymarin, and coumingine HCl, while the tree frog behaves more like the latter.

Our spadefoot toad was secured through Ross Allen's Reptile Institute, Silver Springs, Florida. The mean weight of 69 animals recorded at random was 13.62 ± 0.42 g. The parotoid glands are nearly circular in shape and much less conspicuous as compared with those of common toads such as *Bufo fowleri*. Histologically, they appear to have a morphology similar to the parotoid glands of *Bufo*, except that the acini are fewer in number and smaller in size, as illustrated in Fig. 1.

The secretion of the spadefoot toad can be expressed with a mosquito hemostat. The quantity is small and it is serous in color and consistency. It has a pungent odor not unlike that of fresh grass. It is acid to litmus and irritating to nasal mucous membranes causing incessant sneezing. In view of the fact that only little secretion

---

[7] Gunn, J. W. C., and Epstein, D., *Quart. J. Pharm. and Pharmacol.*, 1932, **5**, 180.

[8] Evans, C. L., *Z. f. Biol.*, 1912, **59**, 397.

[9] Straub, W., quoted by Evans.

[10] Clark, A. J., *J. Pharm. and Exp. Therap.*, 1912-1913, **4**, 399.

[11] Hatcher, R. A., *Am. J. Physiol.*, 1909, **23**, 303.

[12] Gunn, J. A., *J. Pharm. and Exp. Therap.*, 1912-1913, **4**, 225.

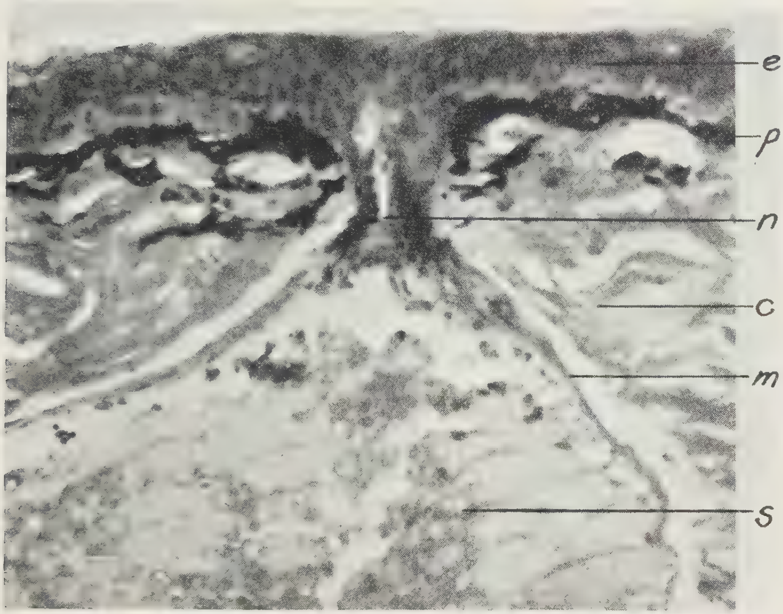


Fig. 1.
Section through a Portion and Duct of a Parotoid Gland.
e. Epidermis; *p.* melanophores; *n.* gland duct; *c.* cutis; *m.* muscular layer; and *s.* secretion.

could be collected by pressure, the glands were carefully excised, thoroughly dried, and pulverized. Exactly one-half gram of the dried glands was extracted under reflux with 30 cc of 95% ethyl alcohol on a water bath for approximately 2 hours. The extract was filtered off, and the process was repeated with a like volume of alcohol for 3 hours. The combined filtrate was evaporated to a small volume, and by addition of 1.4 cc of water, it was brought to a total of 5 cc. The extract gave a positive Liebermann-Burchard's reaction probably due to the presence of sterols such as cholesterol and ergosterol. When injected into the lymph sac or perfused through the inferior vena cava of frogs, no systolic effect could be demonstrated on the heart. This was substantiated in a cat by intravenous injection in which no rise of blood pressure, or bradycardia, or arrhythmia occurred. An alcoholic extract of the parotoid glands of *Bufo* (any common species), similarly prepared, showed a high degree of digitalis-like action. It is thus apparent that the parotoid secretion of the spadefoot toad, *Scaphiopus holbrookii holbrookii*, contains no principles like bufagins or bufotoxins. It, however, appears to have a substance or substances which increase the tone of

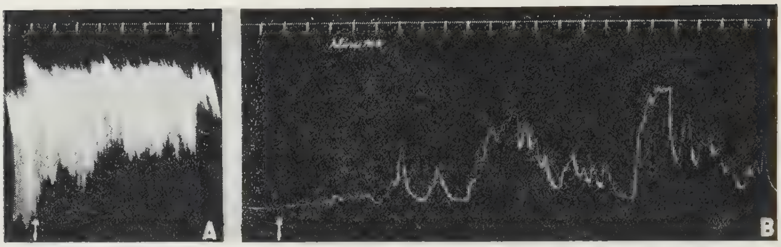


FIG. 2.

Action of the Alcoholic Extract of Parotoid Glands of the Spadefoot Toad.

A. Peristaltic movements of a strip of a rabbit's small intestines immersed in Tyrode's solution at 38°C. At arrow, 0.05 cc of the extract (1%) was applied. Note the increase in tone.

B. Myogram of a horn of a virgin guinea pig's uterus immersed in Tyrode's solution at 38°C. At arrow, 0.5 cc of the extract was added. It resulted in gradual development of contractions.

peristaltic movements of isolated rabbit's intestines and the isolated guinea pig's uterus, as exemplified in Fig. 2.

The tree frog, *Hyla cinerea cinerea,* employed in the present work was purchased from the Southern Biological Supply Company, Inc., New Orleans. The mean body weight of 79 specimens was 5.64 ± 0.103 g. Both the tree frog and the spadefoot toad were subjected to assays with ouabain, cymarin, and coumingine HCl, according to the U.S.P. one-hour frog method,[13] in order to evaluate their sensitivity to these drugs. The results are summarized in Table I and compared with the previously published figures[6] for the Leopard frog, *Rana pipiens,* and the nebulous toad, *Bufo valliceps.* It is obvious that the spadefoot toad is highly resistant to the glycosides, ouabain and cymarin, but slightly less susceptible than the frog to coumingine HCl, the alkaloid having a digitalis-like action. The tree frog is only slightly tolerant to cymarin but not to ouabain or coumingine HCl. No explanation is offered why one amphibian is naturally more sensitive than the other to cardiac poisons, or why the same animal is sensitive to one poison but resistant to others of the same pharmacological class. It is certain, however, that such a natural resistance does not depend upon the ability of the animal to produce digitalis-like substances in the sense of immunity development, for the spadefoot toad is free from these compounds in its venom. Similarly, the rat is very tolerant to digitalis and strophanthin[11, 12] without incorporating any chemical possessing a cardiac action. The secretion of digitalis-like substances in the venom of the nebulous toad *(Bufo valliceps)* must be considered, therefore, accidental, and not respon-

---

[13] *Pharmacopoeia of the United States,* XI Revision, Mack Printing Co., Easton, Pa., 1936, 136.

TABLE I.

Action of Ouabain, Cymarin, and Coumingine HCl in the Spadefoot Toad and the Tree Frog.

Animal	Drug	Solution	Dose, γ per g	No. in systole / No. used	Median systolic dose ± standard error γ per g	Approximate ratio of susceptibility when the frog is unity
Leopard Frog, *Rana pipiens*	Ouabain	1:20,000	*	*	0.6096 ± 0.0096	1
	Cymarin	1:20,000	*	*	0.5999 ± 0.0159	1
	Coumingine HCl	1:2000	*	*	2.733 ± 0.111	1
Nebulous Toad, *Bufo valliceps*	Ouabain	1:250	*	*	48.65 ± 2.86	1:80
	Cymarin	1:50, alcohol 57% by volume	*	*	1050†	1:1750
	Coumingine HCl	1:100	*	*	159.4 ± 9.1	1:58
Spadefoot Toad, *Scaphiopus holbrookii holbrookii*	Ouabain	1:200	10	0/5	45.94 ± 1.28	1:75
			30	0/5		
			40	1/10		
			45	4/10		
			50	8/10		
	Cymarin	1:200, alcohol 15% by volume	10	0/2	41.57 ± 2.30	1:69
			30	0/2		
			35	3/10		
			40	4/10		
			45	7/10		
			50	7/10		
	Coumingine HCl	1:500	4.00	1/5	4.317 ± 0.228	1:2
			4.50	4/5		
			5.00	4/5		
			8.00	2/2		
Tree Frog *Hyla cinerea cinerea*	Ouabain	1:50,000	0.75	0/5	0.850 ± 0.022	1:1
			0.80	1/5		
			0.90	4/5		
			1.00	5/5		
	Cymarin	1:10,000	3.00	0/5	3.943 ± 0.180	1:7
			3.50	1/5		
			4.00	3/5		
			4.50	4/5		
	Coumingine HCl	1:10,000	2.50	0/5	3.485 ± 0.336	1:1
			3.00	1/5		
			3.50	4/5		
			4.00	4/5		
			6.00	4/5		

*See previous report for protocols.[6]

†This figure was taken from the curve at the level of probit 5.

sible for its tolerance to ouabain, cymarin, and coumingine HCl.

Summary. 1. The parotoid secretion of the spadefoot toad *(Scaphiopus holbrookii holbrookii)* has no digitalis-like effect, but it stimulates the isolated rabbit's intestines and the isolated guinea pig's uterus. 2. The spadefoot toad, like the nebulous toad, *Bufo valliceps,* is definitely more resistant to ouabain and cymarin, and only slightly more resistant to coumingine HCl, as compared with the Leopard frog, *Rana pipiens.* 3. The tree frog, *Hyla cinerea cinerea,* is less susceptible to cymarin than the frog, but almost equally susceptible to ouabain and coumingine HCl.

11630

Toxicity of Saline Extracts of Rabbit Uterus After Estrogen Administration.*

BORIS KRICHESKY AND C. D. HONIG.

From the Department of Zoology, University of California at Los Angeles.

In a previous communication it was demonstrated that saline extracts of full term or immediately post-partum rabbit uteri contain a toxic substance lethal to other rabbits when injected intravenously in doses of 6 cc or less.[1] It was also reported that this toxic substance possesses some physiological properties similar to those of histamine.[2]

Among the consistent characteristics of a full term or immediately post-partum rabbit uterus containing the toxic substance, there are: (1) increased color, of vascular origin;[3] (2) increased volume of the entire uterus;[4] and (3) increased amounts of intercellular fluid, or edema.[5] Similar uterine changes after administration of estrogens to non-pregnant animals have been adequately demonstrated by several investigators. Moreover, it has been demonstrated, at least in other species, that estrogens are present in both blood and urine in increasing amounts as pregnancy progresses.[6] The presence of high concentrations of estrogens in the final stages of pregnancy, and the similarity of uterine changes at term and after administration of estrogens, suggests the possibility that the primary female hormone may bear some relationship to the presence of a toxic substance in uterine tissues. For these reasons it was considered advisable to determine if the toxic factor can be recovered from uteri of non-pregnant rabbits previously injected with estrogens.

---

* Aided by a research grant from the University of California. The stilbesterol was generously supplied by the Eli Lilly and Company, Indianapolis.

[1] Krichesky, B., and Pollock, W., *Science*, 1940, **91**, 410.

[2] Krichesky, B., and Pollock, W., *Am. J. Physiol.*, 1940, in press.

[3] Markee, J. E., and French, H. M., *Anat. Rec.*, 1934, **58**, Suppl., p. 79.

[4] Markee, J. E., and Andersen, E., *Anat. Rec.*, 1934, **58**, Suppl., p. 78.

[5] Krichesky, B., unpublished.

[6] Frank, R. T., *Glandular Physiology and Therapy*, Chap. 16, Amer. Med. Assn., Chicago, 1935.

Ten adult non-pregnant rabbits were treated as follows: (1) 4 animals received theelin; (2) 3 received Progynon B; and (3) 3 received stilbesterol. These hormones were given subcutaneously in doses indicated in the table. Forty-eight hours after the last injection the animals were sacrificed, the uteri excised, and extracts prepared as described in an earlier communication, so that one cc of extract represented 0.5 g fresh uterine material.[2] Four non-pregnant untreated animals provided uteri serving as controls.

TABLE I.
Toxicity of Saline Extracts of Uteri from Oestrogen Treated Non-pregnant Rabbits.*

Animal No.	Material injected	Dosage per injection	No. of injections	Wt of excised uterus g	Avg lethal dose cc
1T	Theelin	100 I.U.	9	10.2	0.5
2T	,,	100 ,,	7	9.5	0.4
3T	,,	100 ,,	5	11.1	0.7
4T	,,	50 ,,	8	9.2	0.9
1B	Progynon B	100 ,,	9	11.0	0.4
2B	,, ,,	100 ,,	9	9.7	0.7
3B	,, ,,	50 ,,	7	9.8	0.5
1S	Stilbesterol	0.5 mg	10	9.8	0.2
2S	,,	0.5 ,,	7	12.0	0.5
3S	,,	0.5 ,,	5	10.2	0.3

*See text for description of controls.

Toxicity of each extract was determined by intravenous injection into each of 3 rabbits weighing between 2.2 and 2.6 kg. The lethal dose, as given in the table, is the average dose required to kill the 3 test animals. Injections were made slowly, at the rate of 0.3 cc per minute, until the animal began to struggle. If the animal succumbed within 2 minutes the amount injected was considered a lethal dose. Each control extract, prepared from normal untreated non-pregnant rabbit uterus, was injected into 2 rabbits. These animals were given a single continuous injection of from 12 to 20 cc with no fatalities.

The data contained in the table indicate that in every case uteri from estrogen-treated animals contained the toxic factor. Extracts from these uteri were more toxic than similar extracts from full term or immediately post-partum uteri.[2] These results indicate that in the rabbit estrogenic material is concerned in uterine mobilization or production of the lethal factor.

11631

Degree of Compensatory Renal Hypertrophy Following Unilateral Nephrectomy.* III. Influence of Testosterone Propionate.

Eaton M. MacKay.

From the Scripps Metabolic Clinic, La Jolla, California.

Selye[1] has found the administration of testosterone propionate to lead to an increase in the size of the kidneys in the mouse. He found that in the rat the kidneys were affected in the same way but to a lesser degree. The report presented here is concerned with the influence of the male sex hormone on the degree of compensatory renal hypertrophy following unilateral nephrectomy. The effect of castration was also observed.

The experimental details are similar to those of related studies.[2,3] The diet was composed of Casein 20, sucrose 45, brewer's yeast 5, Osborne and Mendel standard salt-mixture 5, cod-liver oil 5 and Crisco 20. In the nephrectomized rats the left kidney was always

TABLE I.
Influence of Gonadectomy and Administration of Testosterone Propionate upon Degree of Compensatory Renal Hypertrophy in Male Rats.

	Untreated		Gonadectomized		Testosterone treated	
	Controls	Nephrectomy	Controls	Nephrectomy	Controls	Nephrectomy
Body weight						
Initial, g	317	320	308	313	314	314
Death, g	364	344	319	300	332	321
Body surface						
Sq. dm.	5.8	5.6	5.3	5.1	5.4	5.3
Heart weight						
Actual, mg	991	938	895	798	982	890
Per sq.dm, mg	171	168	169	157	180	167
Right kidney weight						
Actual, mg	1021	1279	913	1161	1029	1486
Per sq.dm.	177	229	172	232	188	280
Per 100 mg heart	103	136	102	145	105	167
*Food intake, g	10.0	9.2	10.0	8.6	10.1	10.5
†Compensatory renal hypertrophy, %		29.4		34.9		48.9

*Average in g per rat per day for the 10-day period preceding death.

†The degree of compensatory renal hypertrophy is calculated from the kidney weight in relation to body surface.

---

* We are indebted to the Schering Corporation, Bloomfield, New Jersey, for the supply of testosterone propionate ("Oreton") used in these experiments.

[1] Selye, H., *J. Urol.*, 1939, **42**, 637.

[2] MacKay, L. L., and MacKay, E. M., *Am. J. Physiol.*, 1927-28, **83**, 179.

[3] MacKay, E. M., MacKay, L. L., and Addis, T., *J. Exp. Med.*, 1932, **56**, 255.

removed. In the gonadectomized groups the rats were castrated at the same time. The hormone treated rats were given testosterone propionate subcutaneously in a concentration of 2.5% in sesame oil. Each rat was given 12.5 mg on the *1st, 4th* and *6th* and 25 mg on the *14th* and *29th* day after the beginning of the experiment. Thirty-four days after the nephrectomies the rats were sacrificed and the degree of compensatory renal hypertrophy determined. All of the animals were of the same age and paired litter mates. There were 4 male rats in each group and since there was no overlapping of the individual figures presentation of the averages will suffice.

The average data in Table 1 suggests no effect of castration on the size of the kidneys in the 2 kidney control rats or the degree of compensatory renal hypertrophy. The testosterone propionate in the doses used did not cause a significant enlargement of the kidneys in the control rats but did cause a very definite increase in the degree of compensatory renal hypertrophy following unilateral nephrectomy. This is of interest in itself and there is the possible applied aspect because the outcome of the damaged kidney in Bright's Disease depends upon the hypertrophy of those renal units which have not been destroyed.[4] Such an application of hormone therapy to improve kidney function would be possible only if the testosterone exerts a specific physiological trophic influence on kidney tissue.[1] Functional studies on larger animals will be necessary to determine this.

Summary. Gonadectomy exerted no influence on the degree of compensatory renal hypertrophy in the male albino rat. Testosterone propionate administration produced a marked increase in compensatory renal hypertrophy.

---

[4] Addis, T., *J. Urol.*, 1939, **41**, 126.

11632 P

Immunological Specificity of Sulfonamide Azoproteins.

A. G. Wedum.

From the Department of Bacteriology, College of Medicine, University of Cincinnati.

The immunological aspect of the toxic manifestations[1, 2, 3] occurring during the course of sulfonamide therapy has been little studied. There are reports that in guinea pigs there can be obtained cutaneous sensitivity to prontosil,[4] but not to sulfanilamide.[5]

There have been a few instances in which hypersensitive patients have shown positive homologous skin tests.[6–10] The phenomenon of photosensitization[1, 3] is well established and there is evidence[11] for an allergic mechanism. However, usually the patch, scratch, and intradermal tests have been negative.[5, 7, 12–15] Experiments using the serums of hypersensitive patients have been negative no matter whether the antigen was a saline solution of sulfanilamide[5, 10] or the azoprotein.[16]

Methods. The sulfonamide* azoproteins were prepared essentially

---

[1] Long, P. H., and Bliss, E. A., *The Clinical and Experimental Use of Sulfanilamide, Sulfapyridine and Allied Compounds,* Chapter on Clinical Toxic Manifestations, 1st ed. 1939, The Macmillan Company, New York.

[2] Keefer, C. S., *Cal. and West. Med.*, 1939, **51**, 143.

[3] Mellon, R. R., Gross, P., and Cooper, F. B., *Sulfanilamide Therapy of Bacterial Infections,* 1st ed. 1938, Charles C. Thomas, Springfield and Baltimore.

[4] Nitti, F., and Bovet, D., *Bull. Soc. Chim. Biol.*, 1937, **19**, 837; cited by Long and Bliss.[1]

[5] Hageman, P. O., and Blake, F. G., *J. A. M. A.*, 1937, **109**, 642.

[6] Salvin, M., *J. A. M. A.*, 1937, **109**, 1038.

[7] Goodman, M. H., and Levy, C. S., *J. A. M. A.*, 1937, **109**, 1009.

[8] Myers, G. B., Vonder Heide, E. C., and Balcerski, M., *J. A. M. A.*, 1937, **109**, 1983.

[9] Sézary, A., and Horowitz, A., *Bull. et mém. Soc. Méd. d. hôp. de Paris,* 1936, **52**, 767; cited by Mellon, Gross, and Cooper.[3]

[10] Loveman, A. B., and Simon, F. A., *Arch. Dermat. and Syph.*, 1939, **40**, 29.

[11] Epstein, S., *J. Invest. Dermatology,* 1939, **2**, 43.

[12] Harvey, A. M., and Janeway, C. A., *J. A. M. A.*, 1938, **109**, 12.

[13] Beinhauer, L. G.; cited by Mellon, Gross, and Cooper.[3]

[14] Steven, R., *Cal. and West. Med.*, 1939, **50**, 419.

[15] Erskine, D., *Brit. Med. J.*, 1939, **2**, 104.

[16] Schlesinger, E. R., and Mitchell, W. L., *Am. J. Dis. Child.*, 1938, **56**, 1256.

* Sulfanilamide, sulfapyridine, 4-sulfanilamidoaniline, sodium sulfathiazole, sulfanilic acid, sulfanilylhydroxylamine. For the first three compounds I am indebted to the American Cyanamid Company.

according to the method of Landsteiner and van der Scheer.[17] The immunizing antigens were made with sulfonamides coupled to beef serum. Test antigens were made with sulfonamides coupled to human serum or egg albumin. The usual methods were employed with the sulfonamide azoproteins to produce and test cutaneous and anaphylactic sensitivity in guinea pigs and precipitins in rabbits. Precipitin tests were made by the ring contact method. The attempt to secure cutaneous sensitivity to the simple chemicals uncombined with protein, by a series of intradermal injections in guinea pigs, followed the procedure of Golden and Gierson.[18] Other details will be given in a later publication.

Discussion and Summary of Results. 1. In all 3 types of experiment, skin tests, anaphylaxis, and precipitin reactions, the sulfanilamide and sulfapyridine azoproteins produced heterologous antibodies for each other although the homologous reactions were always stronger. The animals injected with sulfapyridine azoprotein were more reactive to sulfanilamide azoprotein than vice versa. Therefore it might be predicted clinically that patients hypersensitive to sulfanilamide could tolerate sulfapyridine better than those patients sensitive to sulfapyridine could tolerate sulfanilamide. 2. Sodium sulfathiazole azoprotein did not form a precipitate with sulfanilamide or sulfapyridine azoprotein antiserums. This suggests the thiazole may be too far removed from the fundamental sulfanilamide structure to participate clinically in heterologous reactions. 3. The highest precipitin titer found was that of sulfanilic acid azoprotein antigen and sulfanilamide azoprotein antiserum. It seems difficult to account for this as a minor group phenomenon. Is it possible that the sulfanilamide *in vivo* is changed to a structure like sulfanilic acid which then acts as a precipitinogen? 4. There was no evidence that the simple chemicals uncombined with protein could serve either as immunizing or testing antigens. However, 2% sodium sulfapyridine inhibited sulfanilamide and sulfapyridine azoprotein precipitins without affecting the precipitins for beef serum.

---

[17] Landsteiner, K., and van der Scheer, J., *J. Exp. Med.*, 1927, **45**, 1045.

[18] Golden, A., and Gierson, H. W., *J. Immunol.*, 1939, **36**, 277.

11633 P

Physiology of Pure Culture of *Trichomonas vaginalis.* II. Cell Size in Relation to pH.*

Alfred B. Kupferberg. (Introduced by T. L. Jahn.)

From the Departments of Obstetrics and Gynecology and Zoology, State University of Iowa, Iowa City, Iowa.

Several workers have found large variations in the size of *Trichomonas vaginalis* in fresh preparations from vaginal discharges. Powell[1] reported lengths of 10 to 30μ and Wenrich[2] in 2 sets of preparations obtained lengths of 18 to 36 and 12.6 to 27μ. There has been some question concerning the cause of these variations,[1] and it was with the hope of clarifying this situation that this experiment was undertaken.

The materials and culture-methods used in this experiment are the same as those employed by Johnson.[3]

T. vaginalis was grown in media at various pH values and the organisms were measured. Observations were made on the living organisms sealed with vaseline under a microslip supported by glass fragments. The organisms were thoroughly distributed throughout the media before samples were taken. Measurements of length and width were made on the first 35 motile organisms encountered in the field. No measurements were taken of the amoeboid forms which are considered degenerative.[2]

Two-day cultures—The minimal mean size of the organisms (12.4±0.13μ in length and 9.4±0.11μ in width) appeared at approximately pH 5.40. The maximal mean size (15±0.23μ in length and 11.6±0.18μ in width) occurred at pH 7.60. Between these extremes there was a progressive although somewhat irregular increase in size with increase in pH. The extreme range was from 6.4 to 24μ in length and from 4.8 to 19.2μ in width.

Four-day cultures—After 4 days the media which exhibited a pH of 6.64 to 5.15 after 2 days had become more acid (pH 6.44 and

---

* The author desires to express his sincere thanks and grateful acknowledgment to Doctor Theodore L. Jahn for his guidance and helpful criticism during this investigation, and the writing of this paper. The writer is indebted to Mr. Ray E. Trussell who generously contributed the material used in this study.

[1] Powell, W. N., *Am. J. Hyg.*, 1936, **24**, 145.

[2] Wenrich, D. H., The Morphology of *Trichomonas vaginalis.* Volumen Jubilare Pro Prof. Sadao Yoshida, Vol. II, 1939.

[3] Johnson, Garth, in press.

4.93), while the less acid media became slightly more alkaline (about 0.1 pH unit).

The minimal mean size of protozoa (11.4±0.12 by 9.0±0.10μ occurred at about pH 5.10 to 5.17. In more acid cultures the mean size increased slightly to 12.0±0.12 by 10.6±0.11μ at pH 4.93, and in more alkaline cultures (pH values of 6.46 to 7.60) the mean length increased considerably to about 15.0μ. There were slight variations (14.2 to 16.0μ) of the means within this range, but the sizes were significantly greater than those at pH 5.10 to 5.20. The mean width increased more or less progressively from 9.0±0.05 at pH 5.3 to a maximum of about 12.8μ. The extreme range in 4-day cultures was from 6.4 to 25.6μ in length and from 3.2 to 17.6μ in width.

Six-day cultures—After 6 days cultures in the pH range 4.93 to 5.45 had shifted to 4.58 to 5.34. Cultures more alkaline than pH 5.50 did not change significantly. The minimal size (11.6±0.17 by 9.20±0.08μ) occurred at about pH 4.9 to 5.0. The organisms in the more acid cultures were larger (12.5±0.12 by 10.6±0.14μ), and with increasing alkalinity there was a regular and progressive increase in size to a maximum at pH 6.40 to 7.54. Throughout this latter pH range the mean length of the organisms was 15.2μ, and variations of the mean values were very small (15.0 to 15.4μ). The extremes ranged from 6.4 to 27.2μ in length and from 4.8 to 22.4μ in width. The width increased more or less progressively from the minimum of 9.3μ at pH 4.95 to a maximum of 13.0 at pH 7.54.

Johnson[3] found that the maximal growth in 2-day cultures of *Trichomonas vaginalis* (bacteria-free) occurred at pH 5.45 to 5.55, in 4-day cultures at pH 5.50 to 5.80, and in 6-day cultures at pH 5.15.

The present results indicate that at the optimal pH for growth the organisms are smaller than at any other pH. Johnson, in his 4- and 6-day cultures, found that a decided decrease in numbers of the protozoa occurred at suboptimal hydrogen-ion concentrations. Under such conditions the organisms have apparently ceased to carry on their reproductive and normal metabolic processes, and thus have retained approximately the same size as when in an optimal environment after 2 days. The data show that large variations in size comparable to those previously reported from vaginal smears may also occur in bacteria-free cultures under relatively constant environmental conditions.

11634

Blood Sugar, Insulin, and Dextrose Tolerance in Albino Rat Treated with Carbon Monoxide.

Erma Smith* and K. E. Penrod.

From the Department of Zoology, Iowa State College.

Claude Bernard opened the modern history of CO asphyxia with his publication on toxic substances in 1857. He is credited with recognizing that CO displaces the O_2 in oxyhemoglobin, producing COHb. In the same year he published data concerning hyperglycemia and glycosuria induced by CO asphyxia in man: Makami[1] reported similar findings in rabbits, cats and dogs.

Experimental—Albino rats were used throughout the experiments herein reported. The data concern:

1. The relation of the per cent saturation of the Hb with CO to the blood sugar level, subsequent to single exposures to the gas.

2. Insulin tolerance and dextrose tolerance after exposure to CO daily for several weeks.

The sugar analyses were made by the direct microtitration method of Miller and Van Slyke[2] using samples of tail blood. The degree of saturation of the Hb with CO was determined by the method of Sayers and Yant[3] using vena cava blood.

1. Table I shows the blood sugar-COHb relationship.

Pretreatment blood sugar levels in 68 control male rats averaged 100.1±0.05. The blood sugar rises as the COHb increases until at

TABLE I.
Blood Sugar in Relation to COHb.
(Animals exposed once to CO, then sacrificed.)

No. of rats	%COHb	Blood sugar. Mean in mg/100 cc	Range
68	0	100.1 ± 0.05	93.0-109.0
10	10	114.9 ± 1.80	109.7-116.3
12	20	115.8 ± 1.00	111.6-120.2
12	30	117.4 ± 0.50	114.3-121.2
12	40	125.6 ± 1.40	118.2-135.1
12	50	132.0 ± 1.60	123.4-143.9
10	60	154.8 ± 4.10	137.8-162.3
19	85	166.6 ± 2.10	139.0-183.4

---

* Aided by a grant from the Committee on Scientific Research of the American Medical Association.

[1] Mikami, Shozo, *Tohoku J. Exp. Med.*, 1926, **8**, 113.

[2] Miller, Benjamin F., and Van Slyke, Donald D., *J. Biol. Chem.*, 1936, **114**, 583.

[3] Sayers, R. R., and Yant, W. P., U. S. Bureau of Mines, Technical Paper 373.

TABLE II.
Insulin Tolerance in Controls and CO Treated.
Blood sugar readings in mg%.

	No. of determinations	Pre-injection levels	Post-injection levels				
			½ hr	1½ hr	2½ hr	3½ hr	Mean
Control	50	104	74	61	60	93	72±6.6
Actual reading		98	71	60	50	79	65
CO treated							
Initial difference 6 mg% added	50	104	77	66	56	85	71

the lethal saturation, 85% COHb, the mean hyperglycemia is 166.6±2.10.

II. Glucose and Insulin Tolerance. Four-months-old female rats were exposed over periods of 5 to 12 weeks for one hour daily to 0.32% CO-air mixture. This treatment induces a COHb of 60% to 70% which falls to between 5% and 10% within 24 hours. Rats thus treated constantly carry CO in their blood. Polycythemia is induced and the hemoglobin values range from 16 to 20 g per 100 cc of blood.

Insulin Tolerance—Pre-injection blood samples were withdrawn, then 2 units of insulin per kilo of body weight were injected subcutaneously; blood sugar determinations were made at ½ hour intervals for 3½ hours. Since the pre-injection blood sugar values were 6 mg % lower in the CO treated than in the controls, for a true comparison, 6 was added to all readings for CO treated animals. Both readings are shown in Table II.

The number of blood samples per rat was limited because some animals went into insulin coma. The control hypoglycemic average was 72±6.6 mg%. In the CO treated rats it was 65 mg% preceding and 71 mg% after addition of 6 to the initial readings. This variation is within the standard range of deviation mathematically.

Dextrose Tolerance—Initial blood samples were withdrawn, then dextrose in 20% solution was injected intraperitoneally in doses of 2 g per kilo of body weight. Here the difference in pre-injection blood

TABLE III
Dextrose Tolerance in Controls and CO Treated.
Blood sugar readings in mg%.

	No. of determinations	Pre-injection levels	½ hr	1 hr	1½ hr	2½ hr	3½ hr	Total Mean
Controls	50	107	138	151	140	110	105	129±6.9
Actual reading		99	140	158	145	110	105	132
CO treated								
Initial difference 8 mg% added	50	107	148	166	153	118	113	140

sugar levels was 8 mg%, hence we have added 8 to the readings for CO treated animals.

The control hyperglycemic average was 129±6.9 mg%. In the CO treated it was 132 mg%, but after addition of the initial difference it becomes 140 mg%. This difference is well beyond the range of normal variation.

Conclusions. 1. Acute carbon monoxide asphyxia raises the blood sugar in direct proportion to the percentage of COHb induced. 2. Repeated exposure to carbon monoxide seems not to change the insulin tolerance significantly but to reduce the dextrose tolerance below the limits of normal variation. It reduces the fasting blood sugar 6-8 mg below that of untreated litter mates.

11635

Spatial Configuration and Preparation of Canavanine.

J. F. CADDEN. (Introduced by V. du Vigneaud.)

From the Department of Obstetrics and Gynecology, Cornell Medical College and New York Hospital, New York City.

Canavanine is one of the few amino acids whose spatial configuration has not yet been established. Since this compound, found to exist in a free form in the Jack Bean, is a product of nature it would be of interest to establish its spatial configuration by the method of Lutz and Jirgensons.[1] This method is based on the observation of these workers that the naturally occurring amino acids show a change in specific rotation toward the positive with increasing amount of HCl.

Optical rotation measurements were carried out on 0.1 M canavanine solutions with varying molecular equivalents of HCl and NaOH. In Fig. 1 is shown the curve thus obtained. From this one readily sees that there is a decided shift toward the positive with increasing amount of HCl. Dextrorotatory canavanine therefore should be designated as 1(+) canavanine.

Experimental. Preparation of Canavanine. Jack Bean meal is extracted several times with 50% alcohol (by weight). The extracts are concentrated to remove the alcohol and then made up to a volume approximately one-tenth the volume of the extracts with 5% sulfuric acid. To this acid solution is now added sufficient flavianic acid to precipitate the canavanine. The solution is warmed gently to redissolve

---

[1] Lutz, O., and Jirgensons, B., *Ber. chem. Ges.*, 1930, **63**, 448; 1931, **64**, 1221.

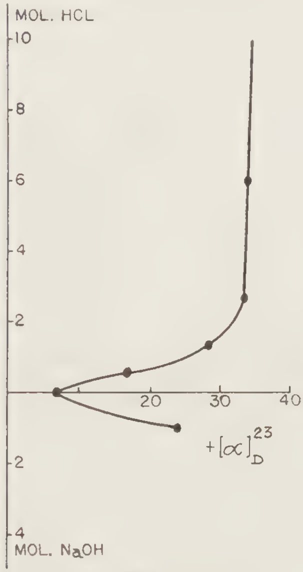


FIG. 1.

The specific rotation of 0.1 M canavanine solutions containing different molecular equivalents of HCl and NaOH.

the precipitate thus formed and then allowed to cool spontaneously. After standing overnight the precipitate is filtered off and washed with cold water. The precipitate is now heated with several portions of 95% alcohol. The alcoholic solutions are combined and allowed to stand overnight. After filtering off the precipitate the filtrate is evaporated under reduced pressure and then on standing a yellow flavianate settles out. The canavanine flavianate thus obtained decomposes at 218-220° C.

Canavanine flavianate is now dissolved in hot water, barium hydroxide added until the solution is definitely alkaline to phenolphthalein. The mixture is allowed to cool, the insoluble barium flavianate filtered off and washed several times with hot water. The filtrate is made acid to congo red with sulfuric acid. To the mixture is added a small amount of norit and after stirring for a few minutes the barium sulfate is removed by centrifugation and filtration. The water clear solution is concentrated under reduced pressure to a small volume. This is added to alcohol with vigorous stirring. The crystals thus obtained are dried by air and then placed in a desiccator. The sulfate decomposes at 172° C.

Canavanine is prepared by adding to a solution of the sulfate exactly the required amount of barium hydroxide, removing the barium sulfate and concentrating the filtrate to a small volume. To the concentrated solution is added alcohol until the solution becomes quite cloudy. On standing in the icebox canavanine crystallizes out. The crystals are washed with alcohol and placed in a desiccator. M. P. 171° C. (decomposition). Although this is a lower decomposition temperature than that reported by Kitagawa[2] and co-workers and by Gulland and Morris[3] who found the free base to decompose at 182° C., a sample kindly furnished me by Dr. Kitagawa behaved in the same manner in my hands as the samples prepared by me.

The values found for carbon and hydrogen 34.14% and 7.01% respectively agree closely with the theoretical values 34.08% and 6.87%. The value found for nitrogen 31.21% was somewhat lower than the theoretical value of 31.81%. It may be noted that the preparation used by Gulland and Morris in their study of the structure of canavanine gave a value of 30.7% for nitrogen. Kitagawa has reported values similar to that obtained by me. However the low nitrogen value does not perceptibly affect the data presented in this report.

Conclusions. The spatial configuration of dextrorotatory canavanine has been determined by the method of Lutz and Jirgensons. It was found to be in agreement with that of the other naturally occurring amino acids and should therefore be designated as 1(+) canavanine. A simplified procedure for the purification of the canavanine flavianate prepared from Jack Bean has been described.

11636

Effect of Subtotal Nephrectomy upon Estrous Cycle of the Albino Rat.

J. T. DIAZ. (Introduced by John C. Burch.)

From the Department of Obstetrics and Gynecology, Vanderbilt University School of Medicine, Nashville, Tenn.

In a previous study of experimental hypertension in the rat,[1] it was found that subtotal nephrectomy was followed by a progressive increase in blood pressure over a period of 3 to 4 months. Daily vaginal smears from a few females that had developed hypertension as a result of this operation revealed marked abnormalities in the

---

[2] Kitagawa, M., *J. Biochem.* (Japan), 1937, **25**, 23.

[3] Gulland, J. M., and Morris, C., *J. Chem. Soc.*, 1935, 763.

[1] Diaz, J. T., and Levy, S. E., *Am. J. Physiol.*, 1939, **125**, 586.

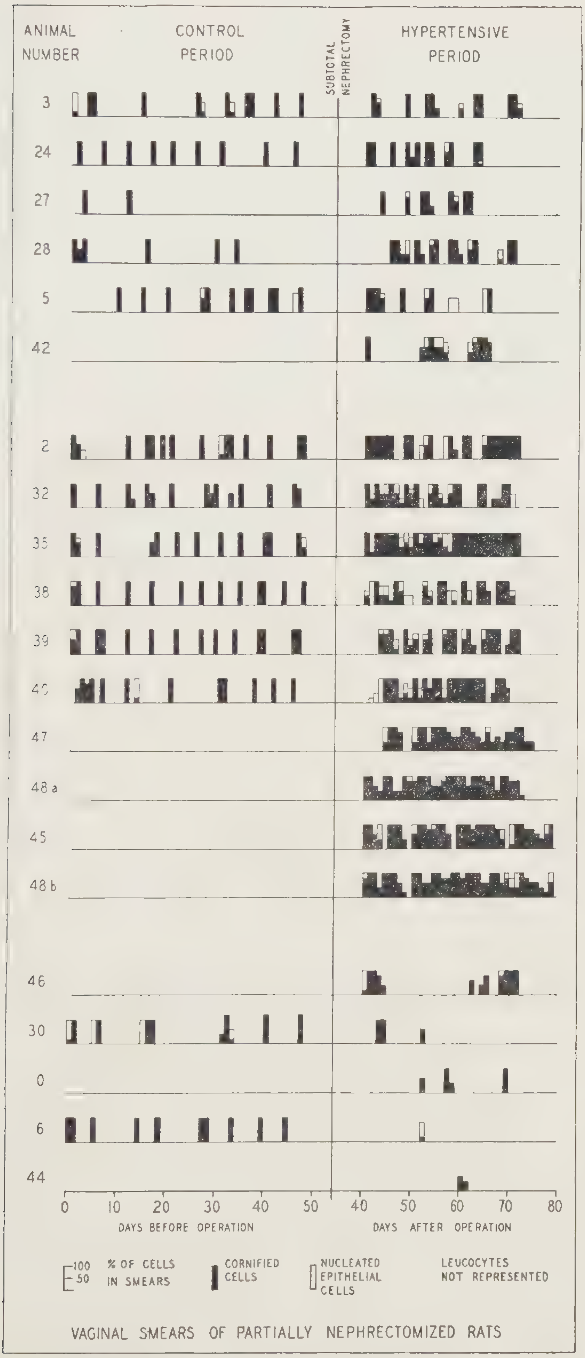


CHART 1.

Vaginal smears of rats subjected to subtotal nephrectomy. Symbols indicate relative proportion of cornified cells and nucleated epithelial cells, respectively. Absence of symbols indicates smear of diestrous type.

estrous cycles. In view of the latter observation, it seemed advisable to obtain additional and more conclusive data on the influence of subtotal nephrectomy upon the estrous cycle. Accordingly, the estrous cycles of 40 adult, healthy female albino rats were followed by means of daily vaginal smears for a control period of 50 days. These animals were then subjected to subtotal nephrectomy by a method described previously.[1] After sufficient time had elapsed for the development of hypertension, vaginal smears were made daily for a period of 35 days. Blood pressures were recorded during the control period and again 3 to 4 months after nephrectomy. Blood pressure was measured by the method of Diaz and Levy.[2] At the end of the experimental period the animals were sacrificed and the weights of the ovaries and pituitaries recorded.

Thirteen of the animals subjected to the above procedures survived

TABLE I.
Blood Pressures and Organ Weights of Rats Subjected to Subtotal Nephrectomy.

Rat No.	Blood pressure* before nephrectomy		Blood pressure* 3-4 mo. after subtotal nephrectomy		Organ wts†	
	Systolic	Diastolic	Systolic	Diastolic	Ovaries	Pituitaries
I 3	110	75	—	—	37.5	6.2
24	120	75	225	125	21.0	4.8
27	105	60	227	125	26.2	7.5
28	120	80	—	85	16.2	4.6
5	120	72	—	—	37.1	6.8
42	110	50	250	140	20.0	3.9
II 2	125	75	220	130	16.6	5.0
32	120	76	220	120	24.5	3.5
35	110	60	—	—	—	—
38	110	78	210	112	15.2	3.1
39	105	58	180	105	14.4	3.7
40	110	75	170	100	11.5	7.0
47	115	60	190	120	21.7	8.6
48a	120	60	260	136	20.0	4.2
45	105	50	210	120	25.0	4.2
48b	120	75	250	160	18.0	4.7
III 46	120	60	270	170	—	—
30	120	85	190	116	26.4	4.1
0	110	74	215	110	28.0	5.3
6	110	65	170	95	12.5	4.6
44	120	55	200	115	—	—
Avgs	114.5	67.5	215.1	121.3		
20 normal rats					26.0	6.8
Group I					26.3	5.63
Groups II and III					19.4	4.8

*In mm of mercury.
†In mg per 100 g body weight.

---

[2] Diaz, J. T., and Levy, S. E., PROC. SOC. EXP. BIOL. AND MED., 1939, **40**, 402.

for 3 to 4 months and remained in good physical condition throughout the experimental period. Observations on these 13 animals and similar observations on 8 rats which were subjected to the same experimental procedures but whose cycles were not observed prior to nephrectomy are presented in Table 1 and Chart 1. The changes in blood pressure (Table 1) were similar to those described previously.[1] Prior to nephrectomy, the estrous cycles were, with few exceptions, normal. During the experimental period, practically all of the animals exhibited abnormalities of the cycle. These abnormalities were of 3 general types, characterized, respectively, by: (1) minor deviations from normal, (2) prolonged periods of continuous estrus, and (3) complete or almost complete absence of estrus (Chart 1). There was no correlation between the type of vaginal disturbance and the degree of hypertension.

These results indicate that disturbances of the reproductive cycle may result from subtotal nephrectomy and, further, that this condition is capable of causing cyclic abnormalities of 3 different types. As a result of studies carried out previously in this laboratory,[3] it has been demonstrated that cyclic disturbances of these same 3 types may result from other and quite different lesions; namely, (1) extreme partial castration and (2) partial hypophysectomy. Observations reported recently by Dey, Fisher, Berry and Ranson[4] indicate that hypothalamic lesions also may give rise to cyclic abnormalities of these 3 types.

From these observations it is clear that functional abnormalities of the female genital tract may result from lesions of the ovaries or from extra-ovarian lesions of either an endocrine or non-endocrine nature, that lesions of a single type may give rise to disturbances of quite different types, and that disturbances of a single type may result from quite different lesions.

---

[3] Burch, John C., McClellan, G. S., Johnson, C. D., and Ellison, E. T., *J. A. M. A.*, 1937, **108**, 96.

[4] Dey, F. L., Fisher, C., Berry, C. M., and Ranson, S. W., *Am. J. Physiol.*, 1940, **129**, 39.

11637

The Breaking Strength of Femurs of Mice Receiving Estrogens.*

W. U. GARDNER.

From the Department of Anatomy, Yale University School of Medicine.

The femurs and other bones, especially of the appendicular skeleton, of mice which received estrogens had greatly thickened walls. Following prolonged treatment the marrow was largely or completely replaced by new osseous tissue.[1, 2, 3] The new bone was well organized and fully calcified. The percentage of ash was increased and the calcium-phosphorus ratio was higher than in the bones of untreated mice.[4] The above observations indicated that the breaking strength of the bones might be increased.

The strength was determined by ascertaining the weight required to fracture the bone. An apparatus was constructed by which weight was progressively and regularly added to the center of a uniform length of bone supported at both ends. Recently dissected femurs were placed over 2 blunted upright steel supports set 1 cm apart. A third freely movable blunted steel knife, attached to a container into which lead shot was delivered, rested upon the bone equidistant between the 2 uprights. The apparatus was so constructed that the addition of shot was prevented as soon as the bone broke. The strength of the bone was determined as the weight in grams required to fracture the femurs. Both femurs of each animal were used.

The breaking strengths of the femurs of 30 estrogen-treated and 9 control mice were determined. The mice were all F_1 hybrids of the C_{57} and CBA strains. They were maintained on a diet of Purina Fox Chow. Weekly subcutaneous injections of 16.6 or 50 μg of estradiol benzoate† were started at weaning and continued throughout life in the test animals. Only 3 mice received the larger amount

---

* This investigation has been supported by the Fluid Research Funds of Yale University School of Medicine, the Jane Coffin Childs Fund for Medical Research, and the Anna Fuller Fund.

[1] Gardner, W. U., and Pfeiffer, C. A., PROC. SOC. EXP. BIOL. AND MED., 1938, **37**, 678.

[2] Gardner, W. U., and Pfeiffer, C. A., PROC. SOC. EXP. BIOL. AND MED., 1938, **38**, 599.

[3] Gardner, W. U., *Anat. Rec.*, 1940 suppl., **76**, 22.

[4] Wentworth, J. H., Smith, P. K., and Gardner, W. U., *Endocrin.*, 1940, **26**, 61.

† The estradiol benzoate (progynon-B) was generously supplied by the Schering Corporation by Dr. E. Schwenk.

of estrogen. The smaller amount was insufficient to result in extreme osseous changes in all of the hybrid mice.

The estrogen-treated mice were killed at ages ranging from 322 to 555 days (average 478) and the controls at ages ranging from 375 to 553 (average 479). The weight required to fracture the femurs under the above conditions ranged from 1599 g to 3542 g (average 2499) for the test animals and 1244 to 2102 g for the controls (average 1655). The breaking strength was closely correlated with the amount of bone present as determined by direct observation and by density as demonstrated by X-ray photographs (Fig. 1). The femurs of 25 of the 30 test mice fractured at weights exceeding that of the strongest control. The average breaking strengths of the femurs of the estrogen-treated mice was over 800 g more than that of the control mice. The average ages of mice of the 2 groups were practically the same.

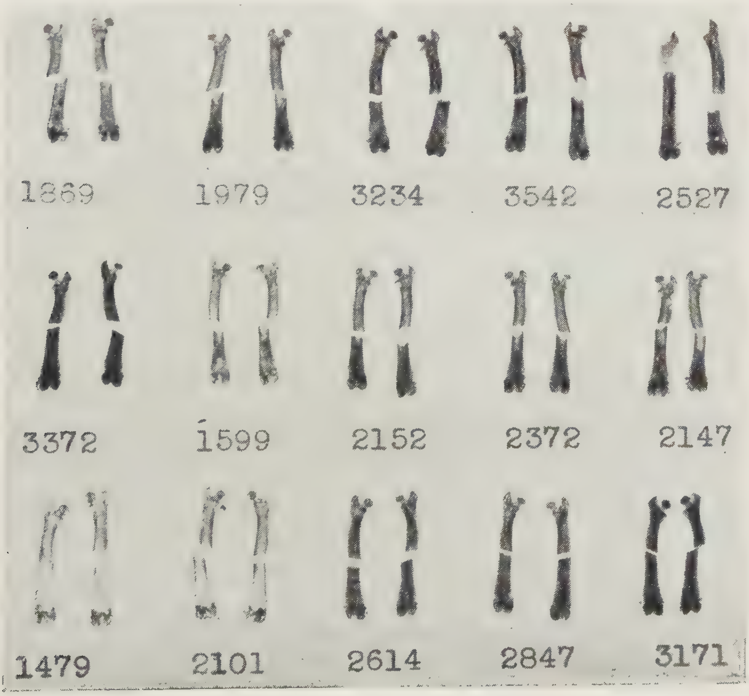


FIG. 1.

X-ray photographs of the femurs of 15 mice (C_{57}♀ × CBA♂). Photographed and printed under identical conditions. The figures indicate the average breaking strengths of the pairs of femurs. The upper 2 rows were from mice which had received 16.6 μg of estradiol benzoate weekly. The 3 groups at the right in the bottom row were from mice which had received 50 μg weekly and the 2 at the left from untreated controls of comparable ages. X-ray photographs taken by Department of Radiology, Yale University.

The weight required to fracture the two femurs of the same mouse varied in most animals. These variations probably represent technical inaccuracies and biological fluctuations. The differences in breaking strength of the 2 femurs of individual estrogen-treated mice averaged 207 g and of the individual controls 147 g, ranging from 0 to 455 and 15 to 275 g.

The length and smallest diameter of the bones were determined by micrometer measurements prior to fracture. The bones of the test mice were approximately 1 mm shorter than those of the controls. The diameters were similar. All the control mice were in excellent general health. The treated mice showed the usual toxic effects of chronic estrogenic stimulation and most of them had tumors of the mammary glands, pituitary gland or both.

Summary. The average breaking-strength of femurs of estrogen-treated mice was 2499 g or 844 g greater than that of untreated controls.

11638

Influence of Sterile Inflammation on Susceptibility to Experimental Poliomyelitis.*

A. F. Rasmussen, Jr., and Paul F. Clark.

From the Department of Bacteriology, Medical School, University of Wisconsin, Madison.

Since neither the serum antibodies nor specific neutralizing substances in the nasopharynx[1] seem to present an adequate basis for the relatively high immunity to poliomyelitis, a further study of the mechanism of this resistance might be made by approaching the problem from the point of view of possible elements, other than the virus, as determining factors in establishing the infection. Is some symbiotic agent, a state of allergy, or an inflammatory condition of the mucosa with resulting increased permeability, a contributory factor in producing clinical cases of the disease?

Flexner and Amoss[2] reported that poliomyelitis could be produced successfully in monkeys with subinfective doses of the virus given intravenously, intranasally, or in the subcutaneous tissues, provided sterile inflammation of the meninges had been incited by the intraspinal injection of normal horse serum or even an isotonic solution such as Ringer's or Locke's solution. They found that a simple lumbar puncture with replacement of the cerebrospinal fluid of one monkey with that from another, or the slightest hemorrhage resulting from the lumbar puncture promoted infection. The controls in their experiments were relatively few, especially when one remembers the inconstancy of the methods of infection employed.

---

* Aided by a grant from the National Foundation for Infantile Paralysis, Inc.

[1] Park, W. H., *J. A. M. A.*, 1932, **99**, 1050; Howitt, B. F., *J. Inf. Dis.*, 1937, **60**, 113.

[2] Flexner, Simon, and Amoss, H. L., *J. Exp. Med.*, 1914, **20**, 249; Flexner, Simon, and Amoss, H. L., *J. Exp. Med.*, 1917, **25**, 1525.

Zwick, Seifried and Witte[3] reported consistent success in the cutaneous infection of rabbits with virus of Borna disease only when injury to the central nervous system had been effected by the injection of non-specific substances such as normal rabbit or normal horse serum.

Sawyer[4] injected sterile starch solution intracerebrally into mice, thereby causing a locus of lowered resistance so that an intraperitoneal injection of a viscerotropic strain of yellow fever virus produced an encephalitis. It has been suggested that the aseptic inflammation produced by these procedures increases the permeability of the blood-brain barrier, thus permitting virus to pass into the nervous tissues.

If an increased susceptibility to intravenous inoculation can be induced by a sterile inflammation of the central nervous system, and if this change is due to an increased permeability of the blood brain barrier, the olfactory tract would not necessarily be involved in such a process. However, Lennette and Hudson[5] have reported that section of the olfactory tracts protected against intravenous inoculation, but that a subsequent intracerebral injection of starch rendered the same animals susceptible to subinfective intravenous doses of virus. These investigators demonstrated poliomyelitis virus in the nasal secretions of one monkey with olfactory tract sectioned about 24 hours after this animal had received virus intravenously, and, on this basis, suggested that in normal animals, even after intravenous inoculation, virus was excreted onto the olfactory mucosa, finally reaching the brain by way of the olfactory nerves. Furthermore, Armstrong[6] presents evidence that treatment of the nasal mucosa with picric acid protects monkeys against intravenous inoculation.

---

[3] Zwick, Seifried, and Witte, *Arch. wissensch. u. prakt. Tierheilk.*, 1929, **59**, 511.

[4] Sawyer, W. A., *J. Exp. Med.*, 1931, **54**, 533.

[5] Lennette, E. H., and Hudson, N. P., PROC. SOC. EXP. BIOL. AND MED., 1936, **34**, 470; Lennette, E. H., and Hudson, N. P., PROC. SOC. EXP. BIOL. AND MED., 1935, **32**, 1444.

[6] Armstrong, Charles, *Pub. Health Rep.*, 1936, **51**, 241.

These findings and hypotheses led us to study anew the relation of sterile inflammation of the central nervous system to the portals of entry in the experimental disease; some of these results were reported briefly at the 1938 meetings[7] of the Society of American Bacteriologists.

Experimental Work: Sterile Inflammation and Infection by the Intravenous Route. In Series I, the olfactory tracts of 4 *Macacus rhesus* monkeys were sectioned surgically by Dr. K. E. Lemmer of the Department of Surgery. After allowing an interval of 3 or 4 weeks for recovery, sterile inflammation was produced in 2 of these animals by injecting 2 cc of sterile horse serum intrathecally and 1 cc of a 2% starch solution into the right frontal lobe of the brain; in Series II and III, 1% starch was injected into both frontal lobes. Each animal then received 10 cc of a 5% brain-cord suspension intravenously on each of 3 successive days, beginning on the day following the injections made for the production of sterile inflammation. Those animals with a sterile inflammation developed the experimental disease, while 2 animals with the olfactory tracts sectioned and 2 normal controls remained well.

A second similar experiment, Series II, with intravenous injection of 55 cc virus suspension, confirmed these results, save that one monkey with sectioned olfactory tracts and with sterile inflammation did not develop poliomyelitis. This animal had been used in Series I, and might, we thought at the time, have had some degree of immunity due to the earlier treatment. We have never found, however, any immunity to actual infection as the result of previous unsuccessful use of monkeys in experimental poliomyelitis, and in this case, subsequent intracerebral injection produced typical paralysis.

The results of these experiments seemed quite clear; an intravenous dose of virus, subinfective for 4 normal controls and 4 animals with olfactory tracts sectioned, incited the disease in 3 out of 4 monkeys with artificial sterile inflammation of the central nervous system even though the olfactory tracts were sectioned.

In the next experiment, Series III, infection by the nasal route was blocked by 4 treatments of the nasal mucosa with 1% zinc sulphate at 12-hour intervals. Twenty-four hours after the last instillation of the zinc salt, sterile inflammation was produced in 2 of these animals and in 2 normal animals by the method described. Upon intravenous inoculation of approximately 10 cc virus suspension per kilo, 3 of 4 animals treated with zinc sulphate developed poliomyelitis; the fourth died an accidental death. One sterile inflammation control, without zinc sulphate treatment, showed no symptoms; the other died an accidental death. Of the normal controls, one developed the disease, the other died of tuberculosis 21 days after inoculation.

---

[7] Clark, Lemmer, and Rasmussen, *J. Bact.*, 1938, **36**, 290.

These results do not agree with the hypothesis that after intravenous inoculation, infection is by way of the olfactory nerve. It is evident that infection by the intravenous route may take place in animals in which the olfactory portal of entry has been blocked either by section of the olfactory tracts or by nasal instillation of zinc sulphate, as has recently been shown also by German and Trask[8] and by Toomey.[9] It is apparent that a sterile inflammation of the nervous tissues decreases the resistance to invasion from the blood stream, but the results reported here emphasize that many of the factors contributing to infection or resistance to infection in the monkey are still unknown. Schaeffer and Muckenfuss[10] have shown that the distribution of virus after intracerebral inoculation is a variable factor which may explain erratic results, particularly when small doses of virus are used. The distribution of starch after intracerebral injection must show a similar variation, with a consequent variation in the degree of injury and inflammation produced. Nor, as King[11] recently emphasized, has it been definitely shown that the inflammatory procedures act solely by increasing the permeability of the blood-brain barrier. The injury to the nerve cells, particularly in Sawyer's method, may be equally important.

The Local Effect of Zinc Sulphate. Schultz[12] has shown that after zinc sulphate treatment the olfactory mucosa is desquamated. Within 4 or 5 days the epithelium regenerates; but it is assumed that the protection, which lasts 8 to 12 weeks, persists until the olfactory nerve endings are regenerated. The protective action is thought to be localized in the olfactory mucosa. If the increased resistance were due to some more deepseated change, it might be expected that inflammation of the central nervous system would alter the effect of the zinc sulphate.

Four monkeys, Series IV, were treated 4 times at 12-hour intervals with about 1.5 cc of a 1% aqueous solution of zinc sulphate introduced a drop at a time. A sterile inflammation was then produced in 2 of these monkeys in the manner described; and all 4, together with normal controls, were inoculated with poliomyelitis virus by dropping about 1.5 cc of a 5% milky virus suspension into each nostril 3 times at 12-hour intervals. All the zinc sulphate-treated animals with or without inflammation, were resistant. Series V is a duplicate of this experiment, giving the same results.

---

[8] German, W. J., and Trask, J. D., *J. Exp. Med.*, 1938, **68**, 125.

[9] Toomey, J. A., *Am. J. Dis. Child.*, 1939, **57**, 338.

[10] Schaeffer, J., and Muckenfuss, R. S., *Am. J. Path.*, 1938, **14**, 227.

[11] King, L. S., *J. A. M. A.*, 1939, **113**, 1940.

[12] Schultz, E. W., PROC. SOC. EXP. BIOL. AND MED., 1938, **38**, 603.

These results tend to confirm the hypothesis of Schultz that the action of zinc sulphate is localized in the olfactory mucosa.

Attempted Infection by the Gastrointestinal Route. The possibility that the increase in susceptibility due to sterile inflammation of the central nervous system might alter the resistance of the monkey to infection by the gastrointestinal route suggested the next experiment, Series VI. To 2 animals with sterile inflammation and to 2 controls, 25 cc of virus were given by stomach tube on each of 3 successive days. All of these animals remained well. As has been pointed out, the increase in susceptibility due to sterile inflammation of the central nervous system may be slight; and hence, this experiment cannot be regarded as definite evidence against the gastrointestinal portal of entry. It shows merely that the inflammation was insufficient to change the monkey's resistance to infection in this experiment.

Summary and Conclusions. 1. Following surgical section of the olfactory tracts or treatment of the nasal mucosa with zinc sulphate, the monkey can still be infected by intravenous inoculation, whether or not a sterile inflammation of the central nervous system is produced. 2. An increase in susceptibility to intravenous inoculation in monkeys with a sterile inflammation of the central nervous system is indicated by the experiments; variation in susceptibility in individual animals is always, however, an important factor. 3. The failure of sterile inflammation of the central nervous system to alter the protective action of nasal treatment with zinc sulphate suggests that the action of zinc sulphate is localized in the olfactory mucosa. 4. The resistance of the monkey to poliomyelitis infection by the gastrointestinal route was not sufficiently altered by a sterile inflammation of the central nervous system to produce the disease.

11639

Antigenic Properties of Living Tissue Cells.

Bjørn Sigurdsson. (Introduced by A. Fischer.)

From the Biological Institute of the Carlsberg Foundation, Copenhagen.

Immune serum against the cells in a tissue culture inhibits their growth completely without necessarily killing them, whereas immune sera against the culture medium, plasma and embryonal extract have no influence on the growth of the cells (Lambert and Haines,[1] Hadda and Rosenthal,[2] Kimura,[3, 4] Craciun and Sorescu[5]). These findings open a new way for investigation into certain biological aspects of the cell that would be accessible by other means only with difficulty.

On immunization with such a complicated structure as a living cell we have to assume that several different antibodies are formed against the different chemical structures. The conditions become even more complicated when we are working not with a pure cellular material but with some crushed tissue containing not only various kinds of cells, but also connective-tissue elements, blood, supporting tissue of various kinds, etc. So the fact that an immune serum, for instance, against minced embryonal material inhibits the growth of homologous fibroblasts *in vitro* gives no exact information about the components taking an active part in the process.

Then the question is: What components of the cells are so vitally important that their blocking by antibodies is incompatible with the growth and proliferation of the cell? Or, in other words: What cellular antigens produce the growth-inhibiting antibodies?

Studies on the properties of the antibody, on the other hand, appear to be of no particular interest. *A priori* it is not reasonable to expect that its nature would differ essentially from that of other know antibodies.

In searching for the active antigens, two procedures are available. One is to treat a fairly large amount of cellular material (*e. g.,* minced embryonal material, "brei") in various ways and then use the purified product for immunization. This procedure has many drawbacks.

---

[1] Lambert, R. A., and Haines, Fr. M., *J. Exp. Med.*, 1911, **14**, 453.

[2] Hadda, S., and Rosenthal, F., *Z. f. Immunitätsforsch. u. exp. Therap.*, 1913, **16**, 524.

[3] Kimura, R., *Z. f. Immunitätsforsch. u. exp. Therap.*, 1928, **55**, 501.

[4] Kimura, R., *Mikrobiologische und immunol. Forsch. unter Anwendung der Gewebezüchtung,* Kyoto, Isseido, 1932, pp. 47-55.

[5] Craciun, E., and Sorescu, *Compt. rend. Soc. de biol.*, 1931, **106**, 761.

For one thing, it requires a rather large amount of material that often is difficult to obtain. Several animals have to be immunized each time, as the animals differ greatly in their reaction to the injected substance. Further, this procedure takes a long time. Besides, and this is even more unfortunate, it gives no information as to the antigenic content of the different products, as the reactive power of the animals is unknown. Last, not least, if there be several different "active" antigens, they cannot be differentiated.

Therefore, the other procedure has been adopted here. The immunization is carried out with untreated cellular material (minced chicken embryo). Then the active serum is absorbed by the product that is to be tested. This procedure has several advantages. It requires but small amounts of material, a few drops or a few centigrams. The absorption takes only a short time. It gives a survey of the active-antigen content of the various preparations, as they may be used for absorption of antibody from the same immune serum (with the same antibody-content), and hence it allows of control series in both directions. If there be several different antigens, they can be differentiated through repeated absorption.

The experiments are carried out in cover-slip cultures with the following medium: 1 drop of chicken plasma, 1 drop of embryonal extract and 1 drop of the serum under analysis. The cultures are made either with fresh heart fragments from a chicken embryo or with older cultures. The latter way offers certain advantages, but it was employed only to a minor extent because it requires a great deal of work to obtain older cultures for all the large experimental series. The size of the heart fragments is an important point. As a rule, complete inhibition of growth from the very small fragments requires but a smaller antibody-content. The fragments must not be loose or frayed; if so, the growth will vary greatly within the same series.

With this procedure the active immune serum obtained by immunization of rabbits with minced chicken embryo inhibits completely the growth of cells in culture. In order to be suitable for absorption it is rather essential that the serum can stand dilution at least to 1:2 (final dilution in the culture 1:6). After being treated in various ways the capacity of the minced embryo for absorption of the active antibody is tried out and then the serum is tested in the cultures. When the absorbed serum is unable to inhibit growth, or when the inhibitory effect is lowered considerably, the conclusion is drawn that the product employed for the absorption has contained unchanged antigen.

It is found that minced embryo after washing with large amounts of physiological salt solution, and long after the biuret reaction in

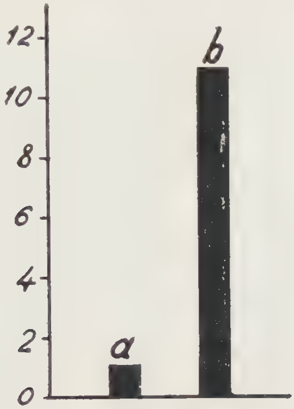


FIG. 1.

Column *a*: Active immune serum, untreated.
Column *b*: Same serum absorbed with washed minced embryo.

This experiment was carried out with cultures in the 6th passage.

The cultures are measured by planimetry at the explantation and after 4 days' growth. The square root of the initial area is subtracted from the square root of the final area, and the difference is recorded on the graph.

Each column presents the average of 5 cultures.

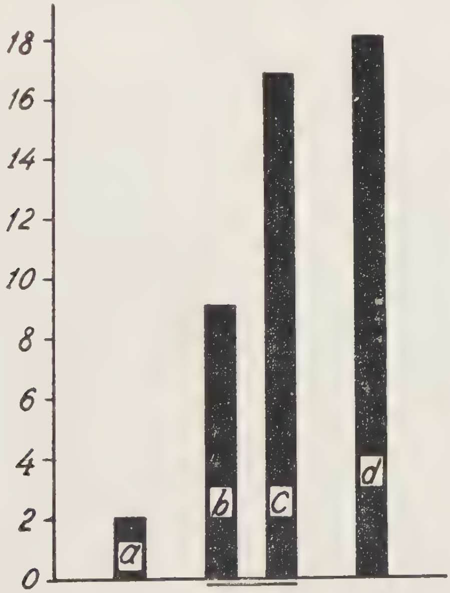


FIG. 2.

Column *a*: Active immune serum, untreated.
Column *b*: Same serum absorbed with hemolyzed erythrocytes.
Column *c*: Normal rabbit serum absorbed in the same manner.
Column *d*: Normal rabbit serum, untreated.

This experiment was carried out with cultures in the 10th passage. Each culture is divided in 4 pieces, one piece for each series. Each column presents the average of 5 cultures.

Measuring and calculation as in Fig. 1.

the wash water has become negative, still is able to absorb the antibody (Fig. 1). Likewise, after standing for a considerable length of time (10-15 days) and repeated washing with physiological salt solution, minced embryo is still capable of antibody-absorption. The same applies to hemolyzed erythrocytes. When employed for immunization the erythrocytes give rise to production of growth-inhibiting antibodies (Kimura[4]), containing thus the active antigen.

I have not been able to obtain any measurable absorption of the growth-inhibiting substance on intact erythrocytes in the proportion of mixture I have employed (1 part 100% erythrocytes + 1 part serum). If, on the other hand, the erythrocytes before this procedure are hemolysed in a hypotonic salt solution, they regularly absorb the antibody in the same proportion of mixture. This prelim-

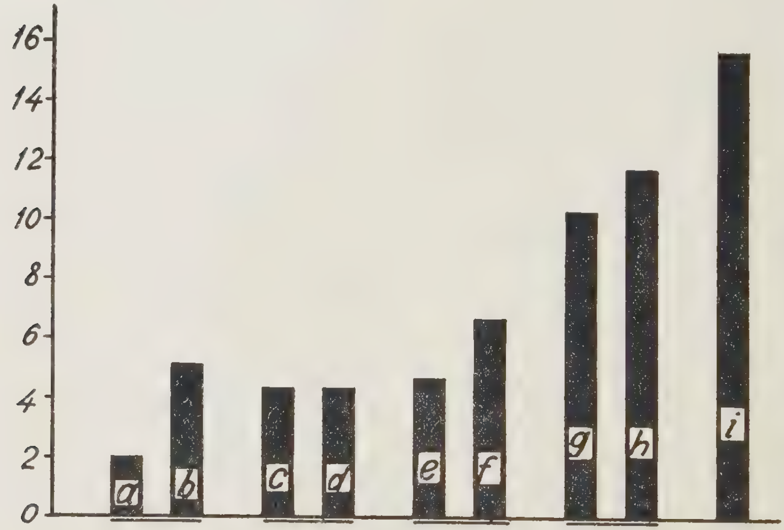


FIG. 3.

Column *a*: Active immune serum, untreated.

Column *b*: Active immune serum, diluted with an equal amount of Ringer's solution.

Column *c*: Active immune serum, absorbed with *washed* minced embryo, 5 drops embryo to 25 drops serum.

Column *d*: Active immune serum, absorbed with *unwashed* minced embryo, 5 drops embryo to 25 drops serum.

Column *e*: Active immune serum, absorbed with *washed* minced embryo, 10 drops embryo to 25 drops serum.

Column *f*: Active immune serum, absorbed with *unwashed* minced embryo, 10 drops embryo to 25 drops serum.

Column *g*: Active immune serum, absorbed with *washed* minced embryo, 25 drops embryo to 25 drops serum.

Column *h*: Active immune serum, absorbed with *unwashed* minced embryo, 25 drops embryo to 25 drops serum.

Column *i*: Normal rabbit serum.

Measuring and calculation as in Fig. 1.

inary hemolysis has to be carried out very gently, however, in order to avoid the formation of clear gelatin-like lumps (nucleic acid?). Serum absorbed on hemolysed erythrocytes shows only a slightly greater fall in hemolytic titer than does serum absorbed on the same amount of intact erythrocytes, but it loses a considerable part of its growth-inhibiting effect.

It seems rather reasonable therefore to think that the interior of the erythrocytes contains the active antigen, at any rate in a larger amount than the surface of the cell, and that this antigen is not identical with the one that gives rise to the formation of hemolysin. But this question requires further investigation.

Minced embryo appears to lose some of its absorbing effect by being washed (Fig. 3).

No evidence was found to suggest that the antigens removed by absorption are qualitatively different from those that remain. Otherwise it is one of the most important questions of the whole problem, whether distinction might be made between different antigens through serial absorption of the same portion of serum by different purified products.

It appears as if washed minced embryo relatively soon loses a considerable part of its absorptive power when it is kept in the icebox. Unfortunately, I have not had time to look into this question systematically. Here, among other things, it is important to ascertain whether the decrease in absorptive power be due to a loss of certain definite antigens or merely a quantitative falling off. Minced embryo triturated with kieselguhr and washed afterwards retains all its absorbing effect. This, together with the experiences with hemolysed erythrocytes, indicates that the "unsoluble" submicroscopic stroma of the cell constitutes the active antigen. If the stroma were a sort of stable inert framework in the cell, one would hardly expect its blocking by antibody to have such a striking effect. Also the lability of the antigen, as manifest on storage of the washed minced embryo, suggests that the stroma is to be looked upon as a functional structure capable of reaction. Here it is to be mentioned, however, that even though the active antibody cannot be absorbed on the surface of the red blood cells, it is by no means established that the inhibition of the growth of the fibroblasts may not be associated with the surface of the fibroblasts. The relation between the amount of intact or living cells in the minced embryo employed for the absorption and its capacity for absorption requires further investigation. By heating at 56° for 30 min. the minced embryo loses its absorptive power. But the immune serum can stand this heating without losing any of its inhibitory effect. Thus complement is not necessary for the inhibition

(Kimura[4]). In undergoing autolysis, by being placed in the incubator at 39° for 5 days, minced embryo loses its absorptive power.

Dr. Albert Fischer has observed that when two tissue cultures are excised and transferred to a tube containing physiological salt solution, they coalesce in their growth, *i. e.*, they grow into each other, and of the two cultures there finally remains only one small spherical block of tissue.

It is found that the anticellular immune serum is able to prevent this coalescence. This observation appears to imply a new way of reaction by which the antibody in the growth-inhibiting immune serum may be titrated. Here it involves a sort of "reverse agglutination." Also fragments of heart may be employed for this reaction, but then the heart has to be incubated previously for 2 hours, or left standing in the refrigerator overnight, as otherwise lymph will exude from the small fragments, and coagulate, preventing thus a close apposition of the fragments. The reaction is carried out in small tubes with a slight diverticulum of the bottom where the fragments are placed so that they can be in contact with each other. The layer of fluid above the fragments should not be too high (0.5 cm), and it is advisable to add a little serum and embryonal extract to the fluid. The size of the fragments plays some rôle too.

Owing to external circumstances my studies had to be discontinued at the present stage. Therefore, I find it appropriate now to make my experiences available to others.

I am greatly indebted to Dr. Albert Fischer for his valuable criticism of my studies as they were progressing.

The work here reported has been carried out by means of support from the Legacy of Consul General Ernst Carlsen and his wife.

11640 P

Nutritional Factors Concerned with Colony Development of *C. Diphtheriae.*

John C. Snyder and J. Howard Mueller.

*From the Department of Bacteriology and Immunology, Harvard University Medical School, Boston, Mass.**

One of us[1] has observed that certain strains of the diphtheria bacillus fail to grow from light inocula on plates prepared from a medium consisting of agar, acid-hydrolyzed casein, nicotinic and pimelic acids, beta-alanine, inorganic salts and a carbon source. Growth sometimes occurs after a lag of 2 or 3 days, sooner as the size of the inoculum increases. Such a medium without the agar, inoculated with a loopful of culture, supports rapid and abundant growth. The addition of horse serum to the agar medium improves it markedly, so that single colonies, (presumably resulting from single organisms or small clumps), appear within 24 hours and at the end of 2 days' growth, attain a considerable size. The horse serum can be diluted, acidified and boiled, and the coagulum removed, the filtrate still possessing the original ability to stimulate prompt growth.

It seems possible that some growth accessory material is involved in the phenomenon which is formed by the organisms themselves, once growth is started. It is carried over in sufficient quantities in large inocula to serve for the initiation of growth, after which, more is formed and development proceeds normally. With a light inoculum this is not the case, and unless the material is supplied in the medium, growth fails.

Further experiments have indicated that the sera of different species of animals do not behave alike. Thus, horse serum can be satisfactorily replaced by ox serum, but not by that of the hog, or by human serum.

If the horse or ox serum be freed as completely as possible from coagulable protein and the clear solution subjected to dialysis through cellophane, or ultrafiltration through collodion membranes, the lag-preventing factor is retained by all but the membranes of relatively large pore size. This fact directed attention to the residual non-coagulable proteins, serum mucoid and the like, but it has not thus far been possible to show any close relationship between these substances and the accessory factor.

---

* Aided by a grant from the Commonwealth Fund.

[1] Mueller, J. H., Proc. Soc. Exp. Biol. and Med., 1939, **40**, 632.

It was then found that when active solutions were concentrated somewhat in vacuum and precipitated by acetone, a separation appeared to take place into two components, neither of which alone was adequate for colony development. When the separate fractions were combined in the original proportions and added to the basic medium, normal growth resulted.

The constant presence of both materials in ox serum suggested the possibility that milk might be found to be a readily available source. Experiment proved this to be the case, and milk appears to be even richer in the factors than serum. Here, however, the behavior is somewhat different in that all the active material is precipitated with the casein upon simple acidification.

Further work on the characterization of the factors is to be reported.[2]

Summary. Rapid development of single colonies of a number of strains of *C. diphtheriae* on a "synthetic medium" agar occurs only in the presence of certain constituents of the blood serum of the horse and ox. Human and swine sera are ineffective. Activity is not impaired by removal of the protein by coagulation. The active material does not dialyze and evidently consists of at least two substances, one of which is soluble in acetone. Cow's milk is rich in these agents, which will be further characterized in subsequent reports.

11641 P

Oleic Acid in Colony Development of *C. Diphtheriae.*

SYDNEY COHEN AND J. HOWARD MUELLER

*From the Department of Bacteriology and Immunology, Harvard University Medical School, Boston, Mass.**

In a preceding note[1] certain properties are described of substances concerned with rapid colony development of strains of *C. diphtheriae*. These occur in horse and ox, but not in human or swine serum. At least 2 compounds are involved, one of which is acetone soluble. Cow's milk is rich in both factors, which are precipitated together with casein upon slight acidification.

---

[2] Cohen, S., and Mueller, J. H., PROC. SOC. EXP. BIOL. AND MED., in press.

* Aided by a grant from the Commonwealth Fund.

[1] Snyder, John C., and Mueller, J. H., PROC. SOC. EXP. BIOL. AND MED., in press.

We have found that ordinary commercial casein provides an abundant source of both substances. One cc of a 10% solution of casein in dilute NaOH added to 15 cc of the agar base† induces rapid growth of colonies with our test strain. The two fractions may be separated by prolonged extraction of the dry casein with hot 96% alcohol, or better, by dissolving the casein in dilute NaOH, reprecipitating it with acid, and repeatedly extracting the moist casein with cold acetone. The present report concerns only the nature of the alcohol and acetone soluble fraction.

Butter is extremely rich in the acetone soluble active material, a few milligrams emulsified in dilute NaOH giving maximal colony development. Saponification of the butter yields a fatty acid fraction containing all the activity. The resulting acids have been esterified and distilled fractionally in vacuum as their methyl esters. When hydrolyzed and subsequently tested, the activity tends to be greatest in those fractions containing the unsaturated acids, particularly oleic acid.

All commercial preparations of oleic acid which we have examined possess activity of about the same order of magnitude as that of the fractionally distilled methyl ester fraction, namely, heaviest growth with about 1 mg per 15 cc of medium. The direct proof of the identity of the growth factor with oleic acid, that is, the use of synthetic material, has not been possible, since the synthesis of oleic acid appears never to have been carried out, and evidently would present considerable experimental difficulty. Certain other evidence, indirect, but chemically fairly conclusive, has been obtained that this acid, rather than some accompanying impurity, is the active agent. These experiments will be detailed in our complete report.

Summary. One of the 2 (or more) factors concerned with colony development, (growth from small inocula) of *C. diphtheriae* appears to be oleic acid. The maximal effect is obtained with about 1 mg in 15 cc of basic medium. The other factor, present in the serum of certain species, in milk, and in commercial casein, is being further investigated.

---

† The basic medium contains an acid hydrolysate of casein, beta-alanine, nicotinic and pimelic acids, a carbon source and inorganic salts.

11642 P

Mammary Carcinomas in Mice Following Oral Administration of Stilbestrol.

Michael B. Shimkin and Hugh G. Grady
(Introduced by Carl Voegtlin)

From the National Cancer Institute, National Institute of Health, United States Public Health Service.

Mammary carcinomas are readily induced by long-continued administrations of large doses of estrogens to mice of susceptible strains. In the numerous investigations[1] on the subject, the subcutaneous route of injection has been used almost exclusively. The induction of breast tumors following oral administration of estrogens has not been reported. The natural estrogens lose most of their estrogenic potency when given by mouth,[2] whereas the synthetic product, stilbestrol (4:4′-dihydroxy-α:β-diethylstilbene) is effective orally,[2] and was used in this experiment.

Forty-one male mice of strain C_3H, raised in this laboratory, were used. Spontaneous mammary tumors develop in practically 100% of the virgin females of this line, but do not occur in the males.[3] The mice were maintained on a standard diet and an unlimited supply of water. They were about 2 months old and weighed from 21 to 23 g when the experiment was initiated.

Stilbestrol* was dissolved in sesame oil, and was fed to the mice by means of a curved metal tube passed into the esophagus. The animals were divided into 4 groups: (I) Eleven were given 0.125 mg of stilbestrol dissolved in 0.1 cc of sesame oil twice a week for 17 weeks, for a total dose of 4.25 mg of the compound. (II) Ten received 0.375 mg of stilbestrol in 0.1 cc of sesame oil twice a week for 17 weeks for a total of 12.75 mg. (III) Ten were fed 0.1 cc of sesame oil twice a week for 17 weeks. (IV) Ten were kept as untreated controls.

The first effect noted was the marked retardation in the gain in weight of the mice which were ingesting stilbestrol (Figure 1). This was not due to loss of appetite, since the consumption of food per gram of body weight was identical in similar groups of mice in which

---

[1] Gardner, W. U., *Arch. Path.*, 1939, **27**, 138.

[2] Leighty, J. A., and Wick, H. J., *Endocrinology*, 1939, **25**, 597.

[3] Andervont, H. B., and McEleney, W. J., *Pub. Health Rep.*, 1938, **53**, 777.

* We are indebted to Merck and Co. for the compound.

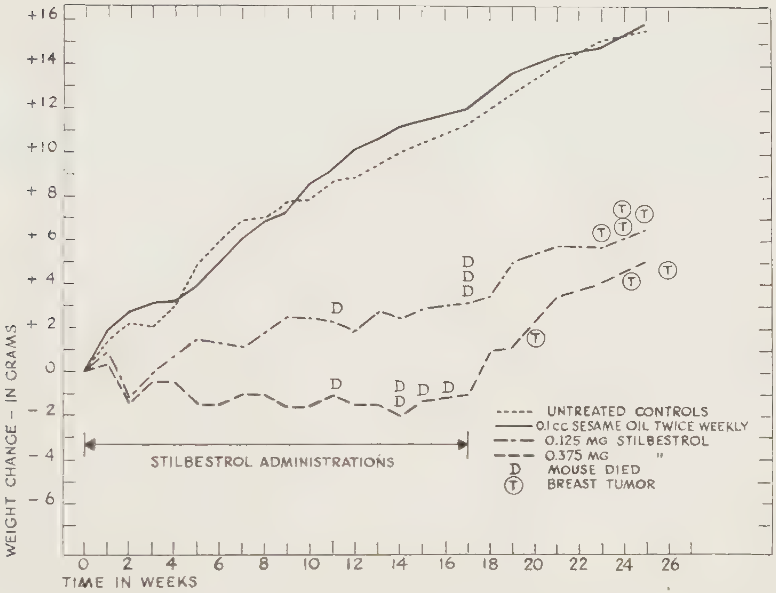


FIG. 1.

Response of male C_3H strain mice to bi-weekly oral administration of stilbestrol.

the food intake was measured for 3 months. The retardation in weight also occurs when the compound is given subcutaneously.[4]

Four of the 11 mice of Group I, and 5 mice of Group II died 11 to 17 weeks after the initial administration of stilbestrol. All showed marked atrophy of the spleen, testes, and seminal vesicles. These atrophic changes were not observed in the mice which developed breast tumors 6 weeks or longer after the cessation of stilbestrol feeding. Presumably such lesions are at least partially reversible if administration of estrogens is discontinued.[5] There was a high incidence of dilatation of the urinary bladder, but death was not attributed to this finding. There were no consistent changes in the gastroenteric tract. No evidence of hepatic damage was seen in these mice, nor in mice of strains C_3H, C_{57} black, C or A which received 5 mg or more of stilbestrol subcutaneously and which have died in the course of experiments now in progress.

Breast tumors, recognized by palpation, began to appear 20 weeks after the initial administration of stilbestrol. Seven mice which received 4.25 mg of the estrogen were alive at this time; of these, 4 have developed tumors, in 23, 24, 24, and 25 weeks. Five mice which ingested 12.75 mg of stilbestrol were alive at 20 weeks; of these, 3 have developed tumors, in 20, 24, and 26 weeks.

---

[4] Gaarenstroom, J. H., and Levie, L. H., *J. Endocrinol.*, 1939, **1**, 420.

[5] Shimkin, M. B., and Grady, H. G., *J. Nat. Cancer Inst.*, 1940, **1**, 119.

All 7 tumors were adenocarcinomas, of the same histologic type as the spontaneous tumors in the females of the strain and the tumors in the males following the subcutaneous injection of estrone or stilbestrol.[5]

11643

Effect of Anti-Placenta Serum on Development of Foetus in the Pregnant Rat.

BEATRICE CARRIER SEEGAL AND EMILY NICHOLS LOEB

From the Departments of Bacteriology and Medicine, College of Physicians and Surgeons, Columbia University, New York City.

This paper reports a series of experiments designed to determine the effect of rabbit anti-rat-placenta serum on pregnancy in the rat. The specificity of the results produced by the anti-placenta serum was controlled by the use of normal rabbit serum, rabbit anti-follutein and anti-pituitary serums, rabbit anti-rat-serum serum, and rabbit anti-rat-whole-blood serum. Another group of pregnant animals received no treatment. The experiments were carried out over a period of 3 years in 2 strains of rats.

Preparation of Antiserums. The method of preparing the rabbit anti-rat-placenta serum was as follows: Pregnant rats were sacrificed one day before term, the placentae were washed in saline, weighed, ground with sand and suspended in saline. The average yield of placental tissue from one rat was 4.5 g; this material was divided into 10 parts and injected intraperitoneally into each of 2 rabbits on 5 successive days. After a rest period of one week the animals were given a second, third and fourth course of immunizing injections and were bled out 10 days later. The serum was inactivated at 56°C for 30 minutes and stored without preservative.

Seven rabbit anti-rat-placenta serums were prepared; the last 3 serums were obtained by immunization with placentae from rats which had previously been perfused with 2 liters of saline, in the manner described by Smadel.[1] The results obtained with all 7 serums were identical.

Two rabbit anti-hormone serums were furnished by Dr. S. C.

---

[1] Smadel, J. E., *J. Exp. Med.*, 1936, **64**, 921.

Werner, one an anti-follutein serum made by immunizing rabbits with Squibb's follutein obtained from the urine of pregnant women, the other an anti-pituitary serum prepared by the injection of rabbits with a pyridine precipitate of the anterior pituitary gland of cattle. An additional rabbit anti-follutein serum was furnished by Dr. H. J. Gegerson.

The anti-rat-serum serum and the anti-rat-whole-blood serum were prepared by injecting rabbits for 5 consecutive days with 0.5 cc either of rat serum or of citrated rat blood. After a rest period of 10 days the injections were repeated for a total of 4 courses. The first course was given intravenously, the remaining 3 intraperitoneally.

Serological Tests. The precipitin content of the serums was determined by diluting the antiserums serially and testing each dilution with antigen diluted 1-20 and 1-100. The presence of lysins was tested for by adding 0.1 cc of increasing dilutions of antiserum to 0.1 cc of 5% rat red blood cells in 0.65 cc of 0.85% saline. After one-half hour at room temperature, 3 units of guinea pig complement in 0.15 cc volume were added. The presence or absence of lysis was noted after 30 minutes at 37°C.

Procedure. The antiserums were injected intravenously into 10-week-old pregnant rats, either of the Sherman strain or of the hooded strain, obtained from Dr. P. E. Smith. The injections were begun routinely on the 11th day of gestation. This was an arbitrarily chosen date since, in a series of 14 pregnant animals in which injections of anti-placenta serums were started variously from the third to the fourteenth day of pregnancy, no difference in results was observed. The injection was repeated on 1 or 2 successive days. In the majority of animals, red blood cell counts were made before injection, 2 days following the last injection and on the 20th day of gestation. The animals were sacrificed on the 20th day of gestation, *i. e.,* one day before the expected date of delivery. At autopsy in each instance the number of normal young as well as the number of placental sites, with or without attached degenerating foetuses and placentae, were noted. Sections of degenerating and of normal placentae, as well as sections of heart, liver, kidney, and uterus, were prepared for histological examination in a representative group of animals.

Results. The series comprises a total of 192 rats. It will be seen from the table that in 68 pregnant rats injected with rabbit-anti-rat-placenta serum, 66% of the developing foetuses were resorbed. Anemia in this group was rare and does not account for the resorption of foetuses observed. At autopsy the only abnormal findings were in

TABLE I.

Effect on Pregnancy in the Rat of the Intravenous Injection of Anti-rat-placenta Serum, Normal Rabbit Serum, Anti-hormone Serum, Anti-rat-serum Serum and Anti-rat-whole-blood Serum.

Type of Rabbit serum	No. of Animals	Avg Dosage, cc	Avg pre-injection RBC count, million per mm³	Avg of lower post-injection RBC count, million per mm³	No. of resorbed young	No. of normal young	% of resorbed young
Anti-rat placenta	68	1.4	7.7	6.4	413	212	66
Controls (uninjected)	27	—	7.0*	6.5*	30	202	13
Normal rabbit serum	15	2.0	8.1†	6.7†	12	121	9
Anti-hormone serums‡	17	2.2	—	—	10	149	6
Anti-rat-serum serum	24	0.9	8.4	5.9	57	170	25
Anti-rat-whole-blood serum	41	0.14	8.1	5.2	279	72	79

*Counts available on 11 of 27 animals. First count taken 11th day of gestation. Second count taken at term.

†Counts available on 5 of 15 animals.

‡Two anti-follutein serums; one anti-pituitary serum.

the placentae and foetuses. In those pregnancies which failed to proceed to term the foetus was either completely autolysed and unidentifiable or it was markedly degenerated so that neither grossly nor microscopically could individual organs be recognized. The placentae in such pregnancies were one-half or less the expected size, were brown rather than red and firmer than normal on section. The placentae which showed the more marked gross changes microscopically presented necrosis of chorionic villi, with hyaline thrombi in the sinusoids, and degeneration of syncytial cells with leukocytic infiltration. In placentae less profoundly affected the syncytial cells appeared similar to those in normal placentae at term but there was atrophy of the villi and hyaline deposits. The picture was similar to that which might be expected coincident with the detachment of the placenta.

In contradistinction to the above group, normal rabbit serum and the 3 anti-hormone serums resulted in no interference with pregnancy. The percentage of normally developing foetuses in these animals was actually somewhat better than that in the control uninjected group. The probable reason for this may lie in the fact that among our control group were 4 animals with a spontaneous anemia with red blood cell counts ranging from 5.6 to 6.0 million cells per cubic millimeter. In one of these 4 animals all of the 8 foetuses had been resorbed. In another only 2 of 7 foetuses had developed. The remaining 2 anemic animals had normal young. No explanation for the anemia was found. Smears from the spleen were negative for *Bartonella muris*. A fifth animal of this group which had resorbed all of its 6 foetuses had not had a red blood cell count. Rats whose initial red blood cell counts were lower than 6 million per cubic millimeter were not used for injection of any of the antiserums.

Increasing doses of injected rabbit anti-rat-serum serum produced an anemia which may account for the interference with the normal development of the foetus found in some of the animals injected with this antiserum. On the whole, however, this serum, except in large doses, produced very little interference with pregnancy. Only 25% of the young were resorbed. This is of particular interest since it was found that the titer of precipitins for rat serum was approximately 30 times as great in the anti-serum serum as in the anti-placenta serum. In the former the antiserum diluted 1-30 gave a precipitate with rat serum, whereas the anti-placenta serum failed to precipitate rat serum at any dilution and gave only a trace when undiluted.

The rabbit anti-rat-whole-blood serum injected into 41 pregnant rats proved extremely damaging, inasmuch as it often produced a severe anemia and also resulted in resorption of the foetuses. Seventy-nine per cent of the young were resorbed. As little as 0.1 cc sometimes was capable of producing an anemia of 2.5 million red blood cells. This anemia alone, however, was not accountable for the interference with the development of the foetuses, since 12 animals with lowest counts of 6 million or better had resorbed all their foetuses. The precipitin titer of this serum was comparable to that of the anti-rat-serum serum. This serum lysed rat red blood cells at a dilution of 1-64, whereas anti-placenta serum lysed only at a dilution of 1-2 and no lysins were demonstrable in the anti-serum serum.

Discussion. The experiments have shown that degeneration of the placenta and foetus of the pregnant rat follows intravenous injection of rabbit anti-rat-placenta serum. This observation is in agreement with the early work on the rabbit and guinea pig by Dobrowalski,[2] and with the more recent findings of Cohen and Nedzel[3] in the guinea pig. The specificity of the action of the antiserum on the placenta is in doubt, however, since a rabbit anti-whole-blood serum is many times more effective in producing the same gross result. The part which antibodies against rat red blood cells may play in the reaction is as yet undetermined. Five absorptions of the rabbit anti-rat-placenta serums with washed rat red blood cells have failed to influence the potency of the serum in producing resorption of the placentae. The anti-placenta serums have all been very weak in their content of antibodies for rat red blood cells even before absorption. Furthermore, anti-placenta serums obtained by immunizing rabbits with well perfused rat placentae were not any less capable of causing degeneration of rat placentae than were serums from animals immunized with placentae containing gross blood. Six absorptions of rabbit anti-rat-whole-blood serum with rat red blood cells reduced but did not eliminate their content of antibodies against rat red blood cells, yet the ability of this serum to cause degeneration of placentae and foetuses was apparently reduced by a disproportionately small degree. Furthermore, this anti-whole-blood serum may be given in such small amounts that the effect on the red cells, as determined by red blood cell counts, is absent, while its ability to cause degeneration of the placentae persists. This problem is under investigation.

---

[2] Dobrowalski, M. S., *Bull. Int. d. l'Acad. d. Sci. de Cracovie*, 1903, No. 5, 256.

[3] Cohen, H. R., and Nedzel, A. J., PROC. SOC. EXP. BIOL. AND MED., 1940, **43**, 249.

The effect of anti-whole-blood serum on pregnancy in the rat appears to be independent of its content of precipitins for rat serum. Anti-rat-serum antibodies have not been injurious to pregnancy in the absence of a serious anemia.

The failure of anti-follutein and anti-pituitary serum to affect the course of pregnancy in the rat agrees with the findings of Zondek and Sulman,[4] who tested anti-gonadotropic serums derived from the goat and were unable to interrupt pregnancy in the rat by this method.

Conclusion. (1) Rabbit-anti-rat-placenta serum effectively interferes with the normal development of the placenta and foetus in the pregnant rat. (2) The same result is obtained with much smaller injections of rabbit anti-rat-whole-blood serum. (3) Rabbit anti-rat-serum serum fails to influence pregnancy unless injected in sufficient volume to induce anemia. (4) Anti-hormone serum and normal rabbit serum are without effect on pregnancy in the rat. (5) The immunological factor or factors responsible for the resorption of the foetuses following the injection of anti-placental serum and anti-whole-blood serum are as yet unknown.

---

[4] Zondek, B., Hochman, A., and Sulman, F., Proc. Soc. Exp. Biol. and Med., 1939, **42**, 338.

11644

Quantitative Studies of Direct Serum Bilirubin.

A. Cantarow, C. Wilmer Wirts, Jr.,* and G. Hollander.

From the Department of Medicine, Jefferson Medical College, and the Laboratory of Biochemistry, Jefferson Hospital, Philadelphia, Pa.

In the presence of hyperbilirubinemia, little or no clinical significance is attached at the present time to the qualitative van den Bergh reaction, except in the diagnosis of hemolytic forms of jaundice. However, an immediate direct reaction is obtained at times in patients with cirrhosis and hepatocellular damage, and following relief of biliary obstruction, in the presence of serum bilirubin concentrations within normal limits. The introduction, by Malloy and Evelyn,[1] of a photoelectric method for the exact quantitative determination of "direct" bilirubin raised the possibility that some light might be thrown upon the significance of the direct van den Bergh reaction and the pathogenesis of hyperbilirubinemia in such cases.

Material and Methods. Determinations were made of total and direct bilirubin, readings being made in the case of the latter at 5 and 30 minutes, employing the photoelectric method of Malloy and Evelyn.[1] In addition, the usual qualitative direct van den Bergh reaction was performed in each instance, development of the characteristic color within 30 seconds being regarded as constituting a positive direct reaction. Data reported here include observations made in the following types of cases: (a) 35 subjects with no evidence of hepatic or biliary tract disease; (b) 107 determinations in 40 patients with hepatocellular damage; 33 determinations in 7 cases of cirrhosis of the liver; 19 determinations during periods of complete biliary obstruction in 8 cases.

Results and Comment. The findings are presented in detail in Table 1. In the normal group, with serum biliary concentrations of 0.1-0.8 mg%, the 5 minute direct bilirubin constituted 0-50% and the 30 minute direct bilirubin 0-75% of the total. Excluding the few instances in which there was no direct bilirubin (total bilirubin 0.1-0.2 mg), the ranges of these values were 12-50% and 25-75% of the total, respectively. These values are in substantial agreement with those reported by Malloy and Evelyn in normal subjects (30 minute direct bilirubin, 35-70% of total).

In the patients with cirrhosis, the proportion of direct bilirubin

---

* Ross V. Patterson Fellow in Gastroenterology.

[1] Malloy, H. T., and Evelyn, K. A., *J. Biol. Chem.*, 1937, **119**, 481.

TABLE I.

Total bilirubin	Normal Direct bilirubin				Cirrhosis Direct bilirubin				Hepatocellular Direct bilirubin				Obstructive Direct bilirubin			
	5 min		30 min		5 min		30 min		5 min		30 min		5 min		30 min	
mg	mg	%	mg	%	mg	%	mg	%	mg	%	mg	%	mg	%	mg	%
.1	0	0	.0 -.05	0-50												
.2	.0 -.05	0-25	.0 -.15	0-75												
.3	.05-.1	17-33	.1 -.2	33-67												
.4	.05-.2	12-50	.1 -.25	25-62	.3	75	0.4	100	.1	25	.3	75				
.5	.15-.25	30-50	.2 -.3	40-60	.15-0.4	30-60	.35- .5	70-100	.1- .3	20-60	.2- .5	40-100				
.6	.1 -.2	17-33	.2 -.3	33-50	.2 -0.4	33-67	.4 - .5	67- 83	.4	67	.5	83				
.7	.15-.2	21-29	.25-.35	36-50					.1	14	.4- .6	57-76				
.8	.2 -.25	25-31	.35	44	.5 -0.6	62-75	.6 - .8	75-100	.1- .5	12-62	.4- .6	50-75				
.81- 1.0							.6 - .7	60- 70	.1- .5	11-50	.4- .8	44-80				
1.1 - 2.0					.7 -1.0	54-71	.4 -1.5	33- 86	.2- 1.1	17-73	.5- 1.4	37-100				
2.1 - 5.0							1.8 -2.3	75-100	.4- 3.8	14-77	.7- 4.2	19-85			2.4- 2.8	80-87
5.1 -10.0							4.7	69	2.1- 6.5	36-81	2.6- 6.9	51-86				
10.1 -20									5.5-12.8	54-69	6.0-13.8	59-85	7.6-11.0	56-71	8.3-11.9	60-75
20+													11.5-20.4	57-79	13.2-22.4	66-81

was rather consistently higher than normal (5 minutes, 30-75%; 30 minutes, 33-100%) throughout the range of values for total bilirubin (0.4-10.0 mg %). This was particularly true with total bilirubin values within the limits of normal (30 minute direct, 67-100%). In the patients with hepatocellular damage there was greater variation in the proportion of direct bilirubin (5 minute, 11-81%; 30 minutes, 19-100%). In those with total bile stasis the 5 minute direct bilirubin constituted 56-79% and the 30 minute 60-87% of the total. These data suggest that in the presence of a serum bilirubin concentration within the limits of normal (0.1-0.8 mg) a direct bilirubin content constituting more than 50% of the total at 5 minutes and more than 70-75% at 30 minutes is indicative of hepatic functional impairment.

An attempt was made to ascertain possible differences in the proportion of direct bilirubin in the sera of patients with normal total bilirubin concentrations giving negative direct van den Bergh reactions and those giving positive direct reactions. The data bearing on this point are presented in Table II. There appears to be no significant difference between either the proportions or the absolute amounts of direct bilirubin in the two groups which could account for the striking difference in behavior of the sera in the qualitative van den Bergh reaction. It must be remembered, however, that the latter is read at the end of 30 seconds, whereas the quantitative direct readings are made at 5 and 30 minutes. The absence of significant relationship between the production of the positive direct van den Bergh reaction and either the total bilirubin concentration, the concentration of direct bilirubin or the proportion which the latter constitutes of

TABLE II.

	Positive direct van den Bergh Direct bilirubin				Negative direct van den Bergh Direct bilirubin			
	5 min		30 min		5 min		30 min	
Total bilirubin mg	mg	%	mg	%	mg	%	mg	%
			Cirrhosis.					
.4	.3	75	.4	100				
.5	.15-.4	30-60	.35-.5	70-100				
.6	.2 -.4	33-67	.4 -.5	67-83	.4	67	.5	83
.7								
.8	.5 -.6	62-75	.6 -.8	75-100	.5	62	.6	75
			Hepatocellular Damage.					
.4					.1	25	.3	75
.5					.1-.3	20-60	.2-.5	40-100
.6	.4	67	.5	83				
.7			.4 -.6	57-86	.1	14	.4	57
.8	.2 -.5	25-62	.4 -.6	50-75	.1-.2	12-25	.4-.5	50-62

TABLE III.
Jaundice Following Sulfanilamide.

Days	1	3	4	5	6	8	10	12
Total bilirubin	5.9	4.6	2.9	1.6	1.1	0.8	1.0	0.4
Direct bilirubin (30 min)	.5	.5	.7	.8	.5	.5	.5	.2
Direct van den Bergh Reaction	—	—	+	+	+	+	+	—

the total is illustrated also by data obtained in a patient with jaundice following administration of sulfanilamide.

Conclusions. The chief clinical value of the quantitative determination of direct serum bilirubin appears to lie in the detection of hepatic functional impairment in the presence of a normal serum bilirubin concentration. Values constituting more than 50% of the total with readings at 5 minutes or more than 70-75% with readings at 30 minutes are probably abnormal. There is no apparent significant relationship between the amount or proportion of direct bilirubin and the production of a positive direct van den Bergh reaction in sera with normal total bilirubin content.

11645

Response of *Sulfapyridine-fast* Pneumococci to Sulfathiazole and Sulfamethylthiazole.

L. H. Schmidt, C. E. Claugus and Effie Starks
(Introduced by S. Tashiro.)

From the Christ Hospital Research Institute and the Department of Biochemistry, College of Medicine, University of Cincinnati, Cincinnati, Ohio.

Our experimental studies,[1] as well as those of MacLeod,[2] have shown that inadequate treatment of pneumococcal infections with sulfapyridine leads to development of organisms which are resistant or *fast* to that drug. Whether these *sulfapyridine-fast* pneumococci are equally *fast* to other sulfanilamide derivatives, such as sulfathiazole and sulfamethylthiazole, is a matter of theoretical and practical interest. Consequently, we have studied the reactions of 2 of our *sulfapyridine-fast* strains to these latter drugs.

Essentially identical experiments have been performed with each *fast* strain and with the parent strains from which these *fast* organ-

---

[1] Schmidt, L. H., and Dettwiler, H. A., in press.

[2] MacLeod, C. M., *J. A. M. A.*, 1939, **113**, 1405.

TABLE I.

Response of *Sulfapyridine-fast* and Parent Strains of Pneumococcus to Sulfathiazole and Sulfamethylthiazole.

Type and strain	No. organisms in infecting dose	No. mice infected	Treatment	No. of deaths Days after infection								Avg hr survival of mice that died	30-day survivors	
				1	2	3	4	5	6	7-10	11-30		No.	%
I McGovern *Fast*	560	30	Sulfapyridine	0	29	1	0	0	0	0	0	41	0	0
		30	Sulfathiazole	1	26	3	0	0	0	0	0	40	0	0
		30	Sulfamethylthiazole	0	28	1	1	0	0	0	0	38	0	0
		10	None—Controls	1	9	0	0	0	0	0	0	27	0	0
I McGovern Parent	520	30	Sulfapyridine	0	0	0	0	1	1	1	0	144	27	90
		30	Sulfathiazole	0	0	1	3	1	0	3	0	131	22	73
		30	Sulfamethylthiazole	0	0	0	1	4	0	2	0	141	23	77
		10	None—Controls	3	7	0	0	0	0	0	0	26	0	0
III Wistuba *Fast*	230	30	Sulfapyridine	0	30	0	0	0	0	0	0	39	0	0
		30	Sulfathiazole	1	28	1	0	0	0	0	0	38	0	0
		30	Sulfamethylthiazole	0	28	2	0	0	0	0	0	39	0	0
		10	None—Controls	6	4	0	0	0	0	0	0	27	0	0
III Wistuba Parent	430	30	Sulfapyridine	0	0	0	2	0	0	20	0	167	8	27
		30	Sulfathiazole	0	0	0	3	1	1	18	0	160	7	23
		30	Sulfamethylthiazole	0	0	1	1	0	3	20	2	171	3	10
		10	None—Controls	9	1	0	0	0	0	0	0	23	0	0

isms were derived.* Mice in groups of 100 were infected intraperitoneally, each mouse receiving 10^{-6} cc of a 12 hour blood broth culture. Ten mice from each group were untreated. The remaining 90 were divided into 3 equal groups which were treated with sulfapyridine, sulfathiazole or sulfamethylthiazole†—20 mg doses of the respective drug being administered at 2, 8, 14 and 22 hours after infection and at 8 hour intervals thereafter for either 5 additional days (in the experiments with the parent strains) or as long as the mice survived (in the experiments with the *fast* strains). The drugs were administered as 10% suspensions in 10% acacia.

The results in the accompanying table show conclusively that *sulfapyridine-fast* pneumococci were also equally resistant to sulfathiazole and sulfamethylthiazole. Thus the mice infected with strain McGovern lived an average of 41 hours under sulfapyridine treatment and 40 and 38 hours under sulfathiazole and sulfamethylthiazole treatments. In the experiment with strain Wistuba, the average survival times were 39, 38 and 39 hours under therapy with the respective drugs.

The table shows also that these drugs were essentially equal in effectiveness against infections with the parent strains. This finding, consistent with those of other workers,[3-7] precludes the possibility that either of the organisms was naturally resistant to sulfathiazole or sulfamethylthiazole. It should be noted, however, that each of the 3 drugs had a greater curative action against infections with the Type I strain (McGovern) than against infections with the Type III strain (Wistuba). This difference in type response is in agreement with our earlier observations.[8, 9]

The finding that pneumococci which are *sulfapyridine-fast* are equally *fast* to sulfathiazole and sulfamethylthiazole offers additional proof that the mode of action of these drugs in pneumococcal infections is fundamentally the same. Moreover, it suggests the futility of attempting therapy with a second of these compounds when the pneumococcal infection becomes resistant to treatment with any one of these drugs.

---

* The preparation of these *sulfapyridine-fast* strains has been described elsewhere.[1]

† We are indebted to Merck and Company, Inc., for the sulfathiazole and sulfamethylthiazole used in this study.

[3] McKee, C. M., Rake, G., Greep, R. O., and Van Dyke, H. B., PROC. SOC. EXP. BIOL. AND MED., 1939, **42**, 417.

[4] Cooper, F. B., Gross, P., and Lewis, M., PROC. SOC. EXP. BIOL. AND MED., 1939, **42**, 421.

[5] Barlow, O. W., and Homburger, E., PROC. SOC. EXP. BIOL. AND MED., 1940, **43**, 317.

[6] Long, P. H., and Bliss, E. A., PROC. SOC. EXP. BIOL. AND MED., 1940, **43**, 324.

[7] Litchfield, J. T., Jr., White, H. J., and Marshall, E. K., Jr., *J. Pharm. and Exp. Therap.*, 1940, **69**, 166.

[8] Schmidt, L. H., and Hilles, C., PROC. SOC. EXP. BIOL. AND MED., 1939, **40**, 611.

[9] Schmidt, L. H., Hilles, C., Dettwiler, H. A., and Starks, E., in press

11646

A New Material (Lygranum) for Performance of the Frei Test for Lymphogranuloma Venereum.

ARTHUR W. GRACE, GEOFFREY RAKE AND MORRIS F. SHAFFER

From the Long Island College Hospital and the Department of Dermatology and Syphilology, Long Island College of Medicine, Brooklyn, N. Y., and the Squibb Institute for Medical Research, New Brunswick, N. J.*

No completely satisfactory product for the performance of the Frei intradermal test for the diagnosis of lymphogranuloma venereum has yet been obtained. The materials commonly employed for the purpose are a suspension, in sterile physiological saline, of human pus or mouse brain containing the heat-inactivated virus of the disease. Many critical reports upon the value of these materials, which are termed Frei antigens, have already appeared, *e. g.*[1] It was felt that a suspension of elementary bodies of the virus of lymphogranuloma venereum might be the ideal agent for the performance of the Frei test. Accordingly, this virus was grown in the yolk sac of the developing chicken embryo[2] according to the method of Cox[3] and a considerable yield of elementary bodies obtained. One cc amounts of suspensions of virus-infected yolk sac showing many elementary bodies were inoculated into the yolk sac of eggs of 6 days' previous incubation. As soon as the embryos were moribund or dead the yolk sacs were collected, washed lightly to remove excess yolk and then weighed. They were ground thoroughly in a mortar with sterile quartz fragments and sufficient sterile physiological saline added to make a 10% suspension. This suspension was centrifuged for 1 hour at 2500 RPM in the cold and the sediment discarded. The super-

---

* Aided by a grant from the United States Public Health Service.

[1] Grace, A. W., *Arch. Dermat. and Syph.*, 1939, **39**, 347.

[2] Rake, G., McKee, C. M., and Shaffer, M. F., PROC. SOC. EXP. BIOL. AND MED., 1940, **43**, 332.

[3] Cox, H. R., *Pub. Health Rep.*, 1938, **53**, 2241.

natant was further centrifuged for 2 hours at 12,500 RPM in the cold. The resulting supernatant was discarded and the sediment resuspended in 0.1% formalin-saline to a volume 20 times that of the original 10% yolk sac suspension. Later, phenol to a final dilution of 0.25% was also added. After 2 days the suspension was tested for bacteriologic sterility and for freedom from active virus by inoculation into the brains of mice and into the yolk sacs of developing hen's eggs. This inactivated suspension has been designated Lygranum antigen.[4]

The normal control material was produced in exactly the same manner with the exception that normal yolk sac from 10-day embryos was used as the starting material. This material has been termed Lygranum control.

During the Spring of 1940, tests with Lygranum control and with Lygranum antigen were performed upon 36 subjects. Twenty-six of these presented signs which were compatible with a previous or existing infection with lymphogranuloma venereum in the individual or in the marital partner. This group is referred to in the paper as "clinically lymphogranulomatous". The remaining 10 persons had neither history nor signs of the disease in themselves or in the marital partner.

Each individual was also tested at the same time with suspensions of normal and lymphogranulomatous mouse brain and all tests were performed by the intradermal inoculation of 0.1 cc of material. Exact measurement of both principal diameters of the central papule or indurated area produced by the inoculation was made with a millimeter scale at the end of 48 hours in 18, and at 72 hours in 18 persons. The significant element in the reaction is the size of the central papule or indurated area which is shown in the Table. The size of the accompanying erythematous area is without diagnostic value.

In the Table are shown the results of the tests of Lygranum and mouse brain controls and antigens upon non-lymphogranulomatous and lymphogranulomatous subjects as well as the clinical condition of each individual.

The results of the tests of Lygranum control upon 35 non-lymphogranulomatous and lymphogranulomatous persons and of Lygranum upon 9 non-lymphogranulomatous subjects indicated that the upper limit of the diameter of non-specific papules produced by these materials was 5 mm and that papules of 5 mm or less, as measured on both principal diameters, represented negative reactions. Papules of

---

[4] McKee, C. M., Rake, G., and Shaffer, M. F., Proc. Soc. Exp. Biol. and Med., 1940, **44**, 410.

TABLE I.

Case No.	Sex	Color	Size of papule (mm) produced by				Interpretation of reaction		Clinical condition
			Mouse brain		Lygranum		Mouse brain Antigen	Lygranum Antigen	
			Control	Antigen	Control	Antigen			
					Clinically Lymphogranulomatous Individuals.				
1	M	W	5x5	38x22	Nil	30x30	Positive	Positive	Purulent inguinal adenitis, 1915, abscess of thigh.
2	F	Col	3x3	21x19	Nil	25x22	,,	,,	Active proctitis, rectal stricture, colostomy
3	M	W	6x6	16x15	Nil	15x14	,,	,,	No evidence of lymphogranuloma venereum. Wife has active lymphogranuloma venereum.
4	M	W	5x4	13x11	Nil	14x10	,,	,,	Active proctitis, rectal stricture.
5	F	Col	4x4	12x12	Nil	11x9	,,	,,	Active proctitis, rectal stricture.
6	M	Col	Nil	11x11	Nil	14x12	,,	,,	Active proctitis.
7	M	Col	5x5	11x11	Nil	13x13	,,	,,	Purulent inguinal adenitis.
8	M	W	Nil	11x11	Nil	10x9	,,	,,	Active proctitis, rectal stricture.
9	M	Col	Nil	11x10	Nil	19x15	,,	,,	Purulent inguinal adenitis.
10	M	W	6x6	10x10	Nil	13x12	,,	,,	Active proctitis.
11	M	W	Nil	10x9	Nil	9x8	,,	,,	Perianal fissure.
12	M	Col	3x3	9x9	Nil	7x7	,,	,,	Primary syphilis with inguinal adenitis.
13	M	W	3x2	9x9	Nil	6x6	,,	,,	Generalized adenopathy.
14	M	W	Nil	9x8	Nil	18x13	,,	,,	Inguinal adenitis in 1925.
15	M	Mex	5x3	9x8	4x3	11x9	,,	,,	Healed proctitis, rectal stricture.
16	M	W	6x5	9x7	Nil	7x6	,,	,,	Active proctitis, rectal stricture.
17	M	W	5x4	8x7	Nil	8x7	,,	,,	Active proctitis.
18	M	W	6x6	7x6	Nil	6x6	Doubtful	,,	Active proctitis, rectal stricture.
19	M	W	Nil	7x6	Nil	6x6	,,	,,	Active proctitis, rectal stricture, elephantiasis of penis and scrotum.
20	M	Col	5x4	6x6	Nil	8x7	Negative	,,	Primary syphilis with inguinal adenitis.
21	M	W	4x4	6x6	Nil	7x6	,,	,,	Purulent inguinal adenitis 1920.
22	M	W	4x4	6x6	3x2	7x6	,,	,,	Active proctitis, carcinoma of rectum.
23	M	W	5x4	6x5	Nil	7x5	,,	Doubtful	Perianal abscesses and sinuses.
24	M	W	5x4	6x5	Nil	5x3	,,	Negative	Perianal fissure.
25	M	W	Nil	4x4	Nil	6x4	,,	,,	Active proctitis.
26	F	W	Nil	3x3	Nil	2x2	,,	,,	Active proctitis, rectal stricture.
					Non-Lymphogranulomatous Individuals.				
1	M	W	3x3	6x5	Nil	5x4	Negative	Negative	Pruritus ani.
2	M	W	4x3	5x5	Nil	Nil	,,	,,	Ruptured appendix.
3	M	W	4x4	5x4	Nil	5x4	,,	,,	Periurethral sinus.
4	F	W	3x3	4x4	Nil	Nil	,,	,,	Congestive cardiac failure.
5	F	W	3x3	4x4	Nil	Nil	,,	,,	Constipation.
6	M	Red	3x3	4x4	Nil	4x4	,,	,,	Secondary syphilis.
7	M	Col	3x3	4x3	Nil	5x4	,,	,,	Chancroid.
8	F	W	3x2	3x3	Nil	3x2	,,	,,	Primary syphilis.
9	F	Col	3x3	3x3	Nil	Nil	,,	,,	Ventral hernia.
10	M	W	4x3	4x3	10x10	10x10	,,	,,	Primary syphilis.

diameter 6 mm or larger, produced equally with Lygranum control and antigen in the same person probably represent a reaction of hypersensitivity to chick material. In such instances the lymphogranulomatous status of the individual must be determined by tests with antigens prepared from mouse brains or human pus.

The results of the tests with mouse brain control and antigen were in accordance with the published literature[1] upon this subject. Nonspecific papules of average diameter of 4 mm were produced in 28 of the 36 non-lymphogranulomatous and lymphogranulomatous individuals tested with control material and in all of the 10 non-lymphogranulomatous persons tested with antigen. The criteria for the evaluation of reactions produced with mouse brain emulsions were as follows: positive, a papule 7 mm in diameter or larger; upper limit of negative, a papule 6 mm in diameter.

a. Ten Non-Lymphogranulomatous Subjects Tested with Lygranum Control and with Lygranum Antigen.

Lygranum control produced no papular reaction in 9 persons. Lygranum antigen showed no papular reaction in 4, and papules of maximum and average size of 5 x 4 mm and 4 x 4 mm respectively, in 5 persons.

One non-lymphogranulomatous person showed a reaction that was unusual in that the responses to both Lygranum control and Lygranum antigen were identical and appeared as erythematous papules 10 mm in diameter. There was no pruritus or vesiculation at the sites of the tests nor was there any previous history of hypersensitivity to egg. The reaction persisted for the same length of time as a positive Frei response of the same papule size. The reaction to mouse brain antigen was negative. This unusual result was regarded as a manifestation of abnormal sensitivity to chick material.

b. Twenty-six Clinically Lymphogranulomatous Subjects Tested with Lygranum Control.

Twenty-four persons gave no papular reaction. The remaining 2 individuals developed papules measuring 4 x 3 mm and 3 x 2 mm respectively.

c. Twenty-six Clinically Lymphogranulomatous Subjects Tested with Lygranum Antigen and with Mouse Brain Antigen.

There were 22 positive, 1 doubtful, and 3 negative reactions with Lygranum antigen and 17 positive, 2 doubtful, and 7 negative reactions with mouse brain antigen. Seventeen individuals gave positive reactions with both Lygranum and mouse brain antigen. The 2 doubtful reactions with mouse brain were positive when Lygranum was used. The doubtful reaction with Lygranum was negative with mouse brain antigen. The 3 negative reactions with Lygranum were also negative when mouse material was employed.

The quality of the positive reaction to Lygranum was similar to that produced by mouse brain antigen. The erythematous area and papule were, in most instances, approximately the same size with each antigen in the same person. The papule which was obtained by the use of Lygranum was, however, sometimes a little lower and slightly less erythematous than that given by mouse brain antigen. The duration of persistence of the papules was approximately the same with both antigens in the same person. The exact time taken for a reaction to disappear depended upon the strength of the response —a stronger reaction remaining visible for a longer period than a weaker one.

Conclusions. Lygranum antigen is superior to mouse brain antigen in sensitivity and specificity in the performance of the Frei test for lymphogranuloma venereum. Lygranum control is superior also to mouse brain control in producing non-specific reactions in only 2 of 35 persons as compared with 28 of 36 in the case of mouse brain control. In the group of 36 tested only one individual showed a reaction which could be regarded as resulting from hypersensitivity to chick material.

11647 P

Influence of Local Applications of Potassium Chloride on Action Current of the Mammalian Heart.*

Hebbel E. Hoff and L. H. Nahum (Introduced by J. F. Fulton)

From the Laboratory of Physiology, Yale University School of Medicine, New Haven, Conn.

In experiments previously reported, it was found that application to the surface of the heart (cat, dog, rabbit) of squares of filter paper soaked in M/10 or M/5 KCl solutions, produced changes in the electrocardiogram similar to those which follow various types of injury to the heart, except that the potassium effects quickly subsided following removal of the solution.[1] Generally accepted views of the action of potassium ions in the heart and elsewhere suggest that the

---

* This work was aided by a grant from the Fluid Research Funds of the Yale University School of Medicine.

[1] Kish, B., Nahum, L. H., and Hoff, H .E., *Am. Heart J.*, 1940, **20**, 174.

electrocardiographic changes produced by the application of potassium to the surface of the heart result from the reversible extinction of the action current at the point of application. Potassium chloride has in fact been employed to abolish electrical activity at selected regions in muscle and thus to permit recording of monophasic action currents.[2]

We have performed a series of experiments to determine whether

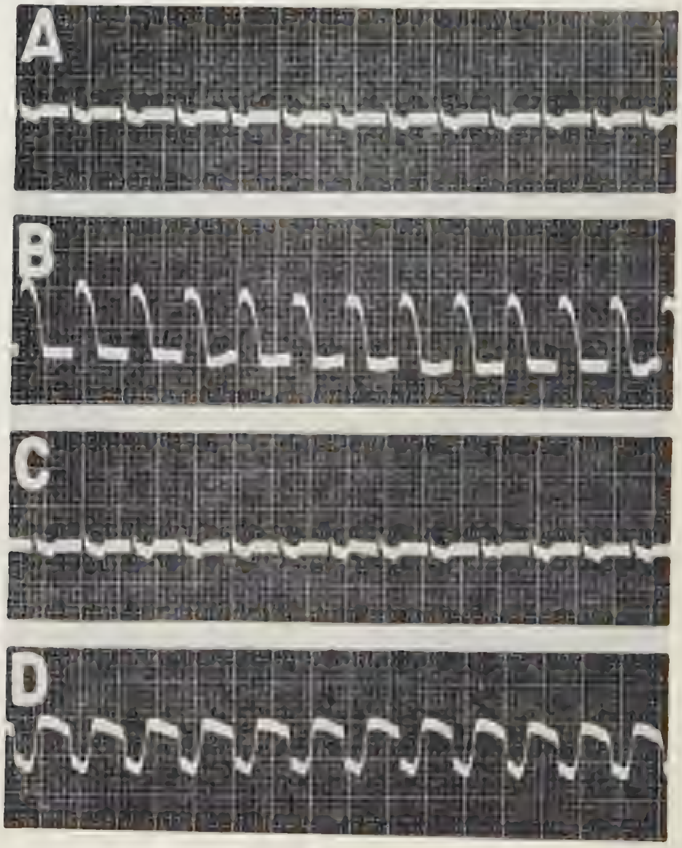


FIG. 1.

Dog 5.5 o, July 15, 1940.

a. Electrogram recorded by electrodes placed on right and left ventricles, anterior surface.

b. Monophasic action current recorded after replacing electrode on left ventricle with wick soaked in M/5 KCl.

c. The potassium electrode on the left ventricle was removed, the surface of the ventricle was washed with Ringer's solution and a new wick soaked in Ringer's solution was replaced.

d. Monophasic curve obtained by replacing lead on right ventricle with wick soaked in M/5 KCl.

---

[2] Buchanan, F., *J. Physiol.*, 1927, **64**, 2.

potassium has a similar effect when applied to the surface of the heart, and found that we could obtain monophasic action currents from the surface of the dog's heart by use of potassium chloride, in M/10, M/5, or isotonic solutions. Electrograms were obtained by wick electrodes soaked in Ringer's solution placed on the anterior surface of the heart, one on the right and one on the left ventricle The wicks led to silver-silver chloride electrodes, and these were connected to a 100,000 ohm potentiometer from which one-fifth of the potential was recorded by an amplifier-type electrocardiograph (Cardiette). Sensitivity was approximately 2 mm per millivolt. After controls were taken, first one and then the other electrode was replaced by a wick soaked in the potassium solution. This procedure yielded monophasic records, and when the leads were arranged so that the spike was upright, the monophasic wave from the right ventricle was upright, while that from the left ventricle was inverted (Fig. 1). Reduction of contact of the heart with surrounding tissues, and slight dryness of the surface of the heart were found to facilitate recording of monophasic waves.

These experiments indicate that potassium chloride solutions of the concentrations employed here create a neutral point at the site of application, and consequently permit recording the monophasic action current indicating the activity at the other electrode. Potassium chloride in proper concentrations, therefore, abolishes electrical activity on the surface of the heart as it is known to do in nerve and other tissues.

Since performing these experiments, we have seen the report of Rothschuh[3] who has obtained similar results.

---

[3] Rothschuh, K. E., *Z. f. d. ges. exp. Med.*, 1939, **106**, 543.

11648 P

Rôle of Liver in Depression of Plasma Potassium Level by Epinephrine.

GEORGE BREWER.

From the Department of Physiology, University of Pennsylvania.

The decline in blood potassium occurring shortly after beginning infusion of epinephrine has been shown not to be due, probably, to depletion of the liver of its potassium stores since the rapid injection of 0.02 mg epinephrine during the infusion transiently raises the blood potassium level.[1] It seems most likely that this decline of potassium level in the blood is due to its entry into the tissues, probably muscle and possibly also back into the liver itself.

In order to investigate this latter possibility epinephrine was infused, over periods up to 45 minutes, into the femoral vein of cats under sodium pentobarbital anesthesia. Blood samples were taken simultaneously from the portal vein and hepatic vein at various times during the course of the infusion. The serum was analyzed for potassium by the method of Kramer and Tisdall;[2] the results are shown in Table I.

TABLE I.
Comparison of Blood Potassium from Hepatic Vein and Portal Vein During Infusion of Epinephrine 1:75,000 at Rate of 1 ml per Minute.

	After 15 min infusion		After 30 min infusion		After 45 min infusion	
Animal No.	Hepatic mg%	Portal mg%	Hepatic mg%	Portal mg%	Hepatic mg%	Portal mg%
1	13.9	16.0	—	—	—	—
2	—	—	12.2	12.5	11.4	12.3
3	10.3	11.7	11.0	12.9	10.0	11.0
4	13.6	13.6	12.4	13.9	14.1	14.3
5	9.0	13.5	12.6	13.9	—	—
6	12.0	14.8	11.3	14.6	—	—

It will be seen that there is almost invariably a difference, small but occurring consistently and probably significant, between hepatic and portal blood potassium concentration. Further, it is the portal blood that contains the larger amount and the hepatic the lesser. This would seem to signify that the liver is removing at least some of the potassium being carried to it by the blood.

---

[1] Brewer, George, Larson, P. S., and Schroeder, A. R., *Am. J. Physiol.*, 1939, **126**, 708.

[2] Kramer, B., and Tisdall, F. F., *J. Biol. Chem.*, 1921, **46**, 339.

It would seem that if the liver is the only source of the potassium mobilized in response to the stimulation of epinephrine[3] it probably continues to release it during the entire time when the epinephrine level of the blood is elevated. However, the liver, as well as the other tissues, very rapidly begins removing potassium from the blood and at such a rate that the blood potassium level is depressed appreciably below the resting normal. The mechanism by which this is accomplished is not clear and whether it depends upon metabolic or purely vascular factors cannot be said at this time.

Summary. During infusion of 1:75,000 epinephrine for as long as 45 minutes at the rate of 1 ml per minute the potassium content of the blood entering the liver, although actually below the resting concentration still exceeds that of blood leaving the liver. This strongly suggests that the cell source from which potassium is mobilized by epinephrine takes part, at least, in removing the excess from the circulation.

11649

Thymus and Lymph Nodes Following Adrenalectomy and Maintenance with NaCl in the Rat.*

W. O. REINHARDT, AND R. O. HOLMES. (Introduced by Herbert M. Evans.)

From the Department of Anatomy and the Institute of Experimental Biology, University of California, Berkeley, California.

The existence of a relationship between the thymus gland and the adrenal cortex has been well established. Adrenalectomy has been reported as causing thymic regeneration,[1] and it has been shown that the inability of the thymus to undergo involution in response to alarming stimuli after this operation is not altered by the administration of sodium chloride.[2] Cortin[3] and adrenocorticotropic hormone[4] have been shown to cause thymic involution. Clinical reports

---

[3] D'Silva, J. L., *J. Physiol.*, 1936, **87**, 181.

* Aided by grants from the Board of Research of the University of California and the Rockefeller Foundation.

[1] Jaffe, H. L., *J. Exp. Med.*, 1924, **40**, 325.

[2] Selye, H., *Brit. J. Path.*, 1936, **17**, 234.

[3] Ingle, D. J., PROC. SOC. EXP. BIOL. AND MED., 1938, **38**, 443.

[4] Evans, H. M., Moon, H. D., Simpson, M. E., and Lyons, W. R., PROC. SOC. EXP. BIOL. AND MED., 1938, **38**, 419.

would seem to indicate a relationship between conditions of adrenal cortical hypofunction and enlargement of thymus and lymphoid tissue. It has been shown in certain species, particularly the rabbit[5] and the rat,[6] that the lymph nodes undergo quantitatively a type of weight involution similar to that of the thymus gland.

In this connection it was of interest to determine whether or not the other lymphoid tissue of the body would exhibit a reaction after adrenalectomy similar to that of the thymus. The use of sodium chloride as a means of prolonging the life of adrenalectomized animals has also prompted an inquiry into the effect of this treatment on thymus and lymphoid tissue of normal and adrenalectomized rats.

Methods. 60-day-old normal male rats of the Long-Evans strain were divided as evenly as possible into the following littermate groups. (1) 60 animals were subjected to a one-stage bilateral adrenalectomy under ether anesthesia using the lumbar approach. Immediately after operation, this group was given 1% NaCl solution as drinking water. Of this number, 44 animals survived the 45-day experimental period and showed no macroscopically visible evidence of adrenal cortical tissue at autopsy. (2) 34 animals were maintained on 1% NaCl solution as salt control animals. (3) 30 animals received ordinary tap water for drinking purposes. All 3 groups were maintained for 45 days under the same experimental conditions, receiving the standard stock diet in use in this laboratory. The animals were autopsied at the age of 105 days at which time they were sacrificed by bleeding under ether anesthesia. The thymus gland of the entire series and the lymph nodes and spleens of representative animals were dissected out, weighed, and fixed for histological study. Dissection of the lymph nodes followed the description given by Job,[7] with some additions, and included cervical, axillary, superior mediastinal, epitrochlear, abdominal and mesenteric groups. All nodes except the mesenteric are grouped under the general heading of systemic nodes. The lymph nodes are constant in position, vary mainly in the subdivisions of the nodes, and are easily dissected. All the nodes and thymus glands were dissected according to a definite routine by the same person to avoid variation.

Results. Table I presents the data obtained.

Inspection of the table shows that the thymus and lymphoid tissues are heavier in the adrenalectomized animals than in the controls.

---

[5] Hellman, T., *Upsala Läk. För.*, Suppl., 1914, May 1.

[6] Chiodi, H., *Endocr.*, 1940, **26**, 107.

[7] Job, T., *Anat. Rec.*, 1915, **9**, 447.

TABLE I.
Thymus and Lymph Nodes.

Type of animal	Thymus			Lymph Nodes			
	No. of animals	Avg B.W.	Thymus wt	No. of animals	Avg B.W.	Systemic nodes	Mesenteric nodes
Normal	20	343	190±12*	7	335	545±40*	366±37*
NaCl Controls	34	340	229±10	6	334	470±32	335±39
Adrenalec. on NaCl	44	303	344±11	11	322	1028±60	440±15

*Weights in mg ± standard error of mean.

The increase in weight is statistically significant as determined by the usual calculations involving standard errors of the differences of the means. Sodium chloride *per se* would seem to cause slight increase in the weight of thymus and the reverse effect on the lymph nodes. The differences are, however, not significant in this number of animals.

It will be noted that the response of the systemic lymph node tissue to adrenalectomy is similar to and even more pronounced than that of the thymus gland. Mesenteric nodes, however, show this response in a more limited manner. The spleen reacts in a manner similar to the thymus and systemic nodes. The figures for this organ are not included because of the incidence of Bartonella infection in the colony.

Histological examination of representative hematoxylin and eosin stained celloidin sections of the thymus and lymphoid tissues of the various groups show that the heavier weight of these tissues in the adrenalectomized animals is due to an actual increase in the bulk of the elements normally present and not to any specific morphological change.

While the above experiments confirm the finding of thymic and lymphoid enlargement noted clinically in Addison's disease and experimentally in adrenalectomized animals, we feel that the use of animals maintained on sodium chloride allows one to study the effects of lack of adrenal cortical hormone uncomplicated by disturbance in electrolyte balance and metabolism as found in the adrenalectomized animal. It should be emphasized that the animals studied after adrenalectomy and maintenance on sodium chloride are to all outward appearances perfectly normal at time of autopsy. The above findings suggest that the thymus gland, systemic lymph nodes, and the spleen react in much the same way to the effects of adrenalectomy. Whether or not this relationship holds true in other experimental conditions is under investigation with the view of determining any further identity of function which may exist in these morphologically related tissues.

Summary. 1. The thymus gland of adrenalectomized male rats maintained for 45 days on sodium chloride does not undergo the involution characterizing the gland in normal untreated animals or those treated with sodium chloride. Sodium chloride as employed appears to exert no consistent effect *per se* on the thymus or lymphoid tissues of the unoperated control. 2. The systemic lymph nodes of adrenalectomized animals maintained on sodium chloride were heavier than those of normal untreated animals or normal animals given sodium chloride. It is notable that the mesenteric lymph nodes do not undergo a comparable gravimetric increase.

11650 P

Effects of Progesterone Upon the Uterus of the Mouse.*

CHARLES W. HOOKER. (Introduced by Edgar Allen.)

From the Department of Anatomy, Yale University School of Medicine, New Haven, Conn.

The fragmentary understanding of the involvement of the corpus luteum in various phases of reproductive activity in the rat and mouse seems in no small measure to be attributable to the inadequacy of the available criteria of activity of the corpus luteum. It seems that no morphologic uterine response to progesterone has been reported for the mouse, and in the rat apparently the only reported effect is mitosis in the epithelium.[1] In the course of a study of the healing of uterine wounds,[2] a definite morphologic response was observed in the uteri of ovariectomized mice receiving progesterone. The experiments described here were conducted to examine the possibility indicated by that observation.

Only young adult mice of the Strong NH strain were used. All the animals were ovariectomized and a period of 3 weeks elapsed between operation and the start of treatment. One group of 6 mice was given daily injections of 0.25 mg of progesterone,† and another group of 6 received 0.5 mg of progesterone daily. Three animals

---

* This study was aided by a grant from the Fluid Research Fund of Yale University School of Medicine.

[1] Hisaw, F. L., Greep, R. O., and Fevold, H. L., PROC. SOC. EXP. BIOL. AND MED., 1937, **36**, 840.

[2] Hooker, C. W., *Anat. Rec.*, in press.

† Supplied by Ciba Pharmaceutical Products, Inc., through the courtesy of Mr. R. C. Mautner.

were given 0.016 mg (500 I.U.) of estradiol benzoate‡ every second day. Three animals received 0.5 mg of testosterone‡ daily. Both progesterone and testosterone in daily doses of 0.5 mg each were given to 2 animals, while 2 other animals were given 0.5 mg progesterone daily and 500 I.U. of estradiol benzoate every second day. The hormones were in solution in sesame oil in such concentration that the injection dose was 0.05 cc. The animals receiving progesterone alone were killed for study after 3 to 6 days of treatment. The remaining animals were autopsied after 5 days of treatment. For comparison 5 animals were killed 26 days after ovariectomy; of these, 4 had received daily injections of 0.05 cc of the bland oil and one was untreated.

Upon gross examination the uteri of the mice given progesterone were slightly larger than the uteri of the untreated animals and were definitely hyperemic. The uteri of the animals receiving this hormone exhibited no contractility upon mechanical or thermal stimulation.

Microscopically the progesterone elicited responses in all the tissues of the uterus. The epithelium was somewhat taller than in the untreated castrates and contained occasional mitotic figures. Mitoses were also present in both layers of the myometrium. The tunica propria presented the most marked changes. This layer was somewhat thickened and slightly edematous, and its blood vessels were dilated and apparently more numerous especially in the basal portion. Numerous mitoses were present—often as many as 10 in a single high power field. The nuclei of the stroma cells had transformed from the rather fusiform, somewhat pyknotic type of the untreated castrate into larger, spherical, vesicular nuclei resembling nuclei of granulosa cells of the ovary. Although these nuclear changes in the stromal cells were the most characteristic response to progesterone. not all the nuclei were thus affected, and in basal portions many nuclei were affected slightly if at all. On the basis of the nuclear changes alone it is impossible to confuse such a uterus with that of an untreated castrate. The smaller dose of progesterone seemed as effective as the larger, and the response was almost as marked at 3 days as after 6 days of treatment.

The uteri of the estrogen-treated animals exhibited the characteristic hyperemia, distention, irritability, epithelial growth, and edema. The nuclei of the stromal cells, although widely separated, were fusiform and pyknotic as in castrates and did not resemble stroma cell nuclei after treatment with progesterone. In the animals receiving progesterone in addition to the estrogen the cellular changes in the tunica propria seen after progesterone alone were added to the typical estrogen effects.

---

‡ Furnished by the Schering Corporation through the kindness of Dr. E. Schwenk.

In the androgen-treated animals the endometrium was more or less intermediate between that of the castrates and of the animals receiving progesterone. The tunica propria was slightly thickened and more fibrous than in the castrates; but the stromal nuclei did not become spherical and vesicular, nor were many mitoses in evidence. In the animals receiving both testosterone and progesterone the effects of both substances were seen in the stroma, although the progesterone characteristics were less marked than in animals receiving progesterone alone.

The tunica propria of the uteri of the few pregnant and lactating mice which have been examined also exhibited nuclei like those described in the castrates receiving progesterone. Apparently, therefore, both endogenous and injected progesterone elicit morphologic change in the uterus of the mouse. Moreover, the nuclear changes in the uterine tunica propria may constitute a specific response to progesterone in the mouse.

Summary. With or without concomitant treatment with estrogen, the daily injection of 0.25 or 0.5 mg of progesterone into mice ovariectomized 3 weeks previously evoked mitosis in all the tunics of the uterus. The nuclei of the cells of the uterine tunica propria became larger and transformed from the fusiform, pyknotic type into vesicular nuclei. The latter condition was also seen in pregnant and lactating mice. Neither estrogen alone nor androgen provoked this morphology.

11651

Effect of Desoxycorticosterone and Testosterone on Water and Chloride Metabolism.

HANS SELYE AND LUCY BASSETT.

From the Department of Anatomy, McGill University, Montreal, Canada.

Selye, *et al.*,[1] showed that high doses of estrogens cause water retention in the rat and this has subsequently been confirmed in the dog by Thorn and Engel.[2] These latter investigators claimed, however, that such an antidiuretic effect is characteristic not only of the estrogens but also of other physiologically active steroid hormones such as the androgens and progesterone. They stated furthermore, that estrone, desoxycorticosterone acetate, corticosterone acetate and androgens decrease the urinary elimination of chlorides, thus the various active steroids appeared to have the same action on water and chloride metabolism irrespective of their specific physiological effects. The subsequent experiments of Selye and Dosne[3, 4] indicated, however, that desoxycorticosterone acetate (D.C.A.) and progesterone differ in their actions on blood chlorides inasmuch as the former elicits pronounced hypochloremia in the rat, while the latter exerts no such effect. Selye and Bassett[5] showed furthermore, that in the rat progesterone causes a pronounced increase in urine output especially after hypophysectomy. In view of these findings we decided to extend our studies and undertake a systematic investigation of the effect of testosterone and D.C.A. on water elimination and chloride metabolism both in the normal and in the hypophysectomized rat.

---

[1] Selye, H., Harlow, C. M., and Collip, J. B., *Endokrinol.*, 1936, **18**, 81.

[2] Thorn, George W., and Engel, Lewis L., *J. Exp. Med.*, 1938, **68**, 299.

In our first experiment 24 female albino rats weighing 91-107 g were divided into 4 groups of 6. Group I was hypophysectomized and received D.C.A. in a peanut oil solution each ml of which contained 20 mg. During the first two days, 2 mg were administered once daily, then the same dose was given twice daily until the thirteenth day, after which 4 mg were injected twice daily subcutaneously, the total dose administered during the 21 days of the experiment being 112 mg per rat. Group II was hypophysectomized but received only as much peanut oil as had to be administered to the treated animals as a solvent for D.C.A. All hypophysectomies were performed on the same day on which the injections were started. Group III was given the same amount of D.C.A as Group I but was not hypophysectomized. Group IV acted as intact controls and received oil only.

All rats were kept in individual metabolism cages and the water intake and urine output were measured daily. The animals were given "purina fox chow" and ground oats *ad lib.* with a few leaves of celery throughout the experimental period except on 3 days on which the effects of changes in food and water intake were studied. Fig. 1, which gives the average water intake and urine output on

---

[3] Selye, Hans, and Dosne, Christiane, PROC. SOC. EXP. BIOL. AND MED., 1940, **44**, 165.

[4] Selye, Hans, and Dosne, Christiane, *Am. J. Physiol.*, in press.

[5] Selye, Hans, and Bassett, Lucy, PROC. SOC. EXP. BIOL. AND MED., 1940, **44**, 502.

the fifth day is representative of conditions as they existed throughout the experiment on days on which water and food were given *ad lib.* It will be seen that D.C.A. markedly increased both the water intake and diuresis but this effect was much more pronounced in the hypophysectomized than in the normal animals.

On the seventh day, food and water was withheld for a period of 24 hours. At the end of this time the normal controls excreted 3.0 ml, the D.C.A.-treated intact animals 7 ml, the hypophysectomized controls 3.5 ml and the hypophysectomized D.C.A.-treated 4 ml of urine on the average. This indicates that in the absence of food and water, the action of D.C.A. was not so obvious as otherwise, especially in the hypophysectomized group; yet the hormone

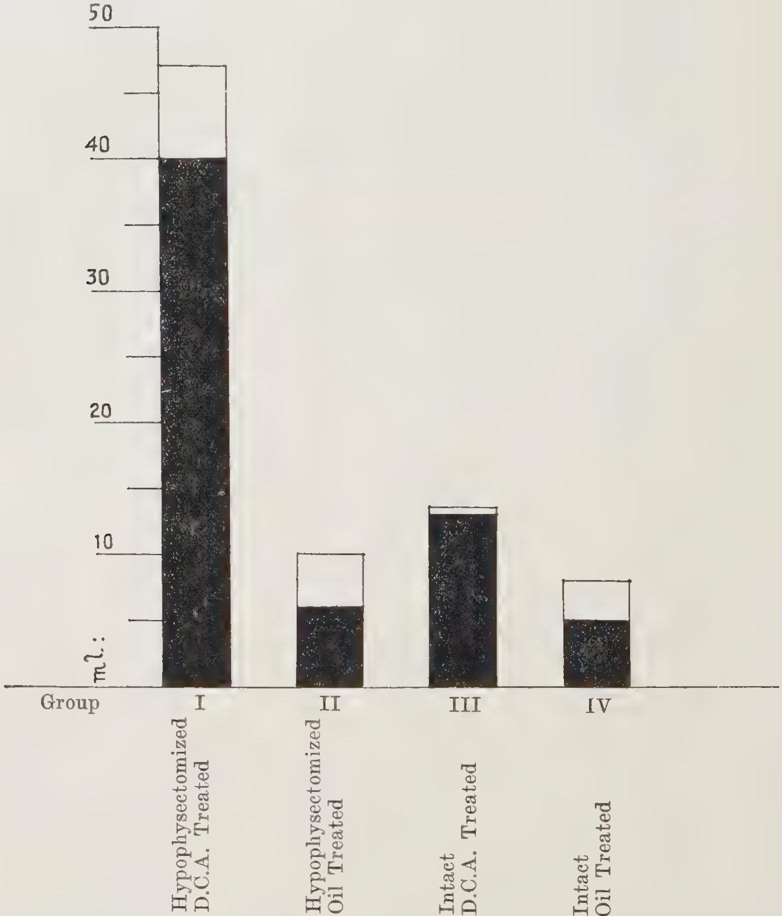


FIG. 1.

Graph illustrating the average urine output of normal and hypophysectomized rats treated with D.C.A. and receiving food and water *ad lib.*

retained some of its diuretic action so that this effect cannot be regarded as a mere consequence of increased water intake. On the twenty-first day the animals were fasted but received 10 ml of water by stomach tube in order to determine whether ingested water is excreted more rapidly by the D.C.A.-treated than by the normal animals. This proved to be the case both in the intact and in the hypophysectomized series so that it may safely be concluded that the action of D.C.A. is not merely due to increased voluntary water ingestion.

The effect of D.C.A. on whole blood and urine chlorides was determined several times during the experiment by the Van Slyke method and Cl is expressed in Table I as NaCl. The figures which appear in brackets in the table indicate the range of the variations in each group. The results given in this table as a representative example were obtained on the 11th day after a 24-hour fasting period so as to avoid post-absorptive variations. Water was allowed *ad lib.* during this period.

As Fig. 1 and Table I indicate, D.C.A. caused marked diuresis both in the normal and in the hypophysectomized animal but this effect was particularly marked in the latter. In some of the D.C.A.-treated hypophysectomized animals, this diuresis may well be compared with that of severe diabetes insipidus. Only average values are given here in order to save space, but we may mention as an example of a particularly severe case that in one animal whose body weight decreased to 90 g after hypophysectomy the urine output reached 85 ml/24 hours on certain days, that is to say, the animal voided almost the equivalent of its own body weight in a single day. The blood chlorides were consistently and markedly decreased by D.C.A. in the intact animals throughout the experiment irrespective of the water intake. No such significant decrease could be obtained, however, after hypophysectomy. The urinary

TABLE I.
Effect of D.C.A. on Urine Volume and Blood and Urine Chlorides of Fasted Normal and Hypophysectomized Rats Receiving Water *ad lib.*

Group Treatment	1 Hypophysectomized D.C.A.	II Hypophysectomized Oil	III Intact D.C.A.	IV Intact Oil
Urine in 24 hr (in ml)	16.8 (14.5-21.5)	4.1 (2.5-5.5)	20.7 (14.5-28.5)	9.9 (4.0-21.0)
Chlorides/100 ml Urine	693 (410-1290)	916 (700-1230)	750 (585-880)	470 (410-585)
Total Urine Chlorides, 24 hr	116 (73.8-187)	37.5 (17.5-51.4)	155 (84.8-199.5)	46.5 (18.8-86.1)
Chlorides/100 ml Blood	423 (386-468)	433 (398-468)	362 (346-374)	441 (416-468)

excretion of chlorides per 24 hours was always greatly increased by D.C.A. irrespective of the presence or absence of the hypophysis. This was due mainly to the increase in urine volume and not to an increased chloride concentration, although on certain days (Table I) the concentration of the chlorides in the urine was also increased at least in intact rats. It will be seen that although in the hypophysectomized animals, D.C.A. failed to decrease the blood chloride concentration, it still increased the total chloride output. The chloride concentration of the urine on the other hand, was not significantly influenced by this steroid. D.C.A. causes marked diuresis even in the hypophysectomized animal in which it does not decrease the blood chlorides. This is in agreement with the finding that progesterone causes polyuria without significantly influencing the blood chloride concentration[5] and indicates that the diuretic effect of these steroids is not dependent upon changes in blood chlorides. The increase in urinary chloride elimination and decrease in blood chlorides reported in this communication are difficult to reconcile with the finding that this same compound causes a rise in blood sodium and a decrease in the urinary sodium excretion.[6, 7] Further experiments will have to show whether these changes in Na and Cl metabolism can be produced with the same dose and at the same time. If this should prove to be the case, it is evident that at least a considerable part of the blood Na and urinary Cl of D.C.A.-treated rats is not present in the form of NaCl.

In connection with these experiments, we should also like to mention that determinations performed on all animals on the last day of the experiment indicate that D.C.A. causes a noteworthy increase in the circulating blood volume both in the hypophysectomized and in the intact animals.

We also studied the actions of testosterone (not esterified) administered in exactly the same manner and dosage as D.C.A. was given in the above experiments. We omit a detailed description of these experiments since testosterone proved to have no effect on diuresis or chloride excretion either in intact or hypophysectomized rats even though such high dosages had been administered. The only significant change observed was a slight decrease in the whole blood chlorides, a fact which is in agreement with the previous observations of Selye and Dosne.[8] These observations clearly indi-

---

[6] Hartman, Frank A., Spoor, H. J., and Lewis, L. A., *Science,* 1939, **89**, 204.

[7] Wells, B. B., and Kendall, E. C., *Proc. Staff Meet. of the Mayo Clin.,* 1940, **15**, 133.

[8] Selye, Hans, et Dosne, Christiane, *Annales d'Endocrinol.,* in press.

cate that there are significant qualitative differences in the action of the various steroid hormones on electrolyte and water metabolism. Since previous observations indicated that androgens, progesterone and D.C.A. cause kidney enlargement due to hypertrophy of the proximal and distal convoluted tubules,[9, 10] we were at first inclined to regard the diuretic action of progesterone and D.C.A. as a consequence of this kidney change. However, the fact that androgens, which are most active in causing renal enlargement, do not elicit diuresis clearly indicates that there is no correlation between the kidney enlarging and the diuretic action of steroids. In conclusion, it may also be mentioned that progesterone, D.C.A., and testosterone all cause significant kidney enlargement even in the hypophysectomized rat but in the latter this effect was much less pronounced than in intact animals. The mechanism of the renal effect will be discussed elsewhere. Suffice it to state here that unlike the mammogenic action of the steroids[11] the renal effect is not entirely mediated by the hypophysis nor is it completely independent of this gland as are the actions of steroids on vagina, oviduct and uterus.[11]

Summary. In the rat, desoxycorticosterone acetate (D.C.A.) causes marked diuresis, accompanied by increased chloride excretion, but not by a constant increase in the chloride concentration of the urine. The diuretic effect is much more pronounced in the hypophysectomized than in the intact rat. Unlike in the intact animal, D.C.A. fails to decrease the blood chlorides in the hypophysectomized rat. The hyperchloruria and polyuria cannot be considered, therefore, as the consequence of the hypochloremia.

The kidney hypertrophy elicited by progesterone, D.C.A., and androgens is likewise not the cause of the polyuria since testosterone —which is most active among the steroids as far as kidney enlargement is concerned—does not influence diuresis. The kidney-enlarging action of all three above mentioned steroids is considerably decreased but not prevented by hypophysectomy.

The expenses of this investigation were defrayed by a grant received by one of us (H.S.) from the Committee on Endocrinology of the United States National Research Council. We are also greatly indebted to Doctors Gregory Stragnell and Erwin Schwenk of the Schering Corporation of Bloomfield, New Jersey, who donated the generous supply of steroid hormones which was necessary for the completion of these experiments.

---

[9] Selye, Hans, *Montreal Physiol. Soc. Meet.*, 1939.

[10] Selye, Hans, *J. Urol.*, 1939, **42**, 637.

[11] Selye, Hans, *Anat. Rec.*, in press.

11652

Carbohydrate Metabolism of the Chick Embryo.

A. J. Dalton and R. F. Hanzal.

From the Department of Anatomy, McGill University, Montreal, Canada, and the Institute of Pathology, Western Reserve University, Cleveland, Ohio.

These investigations are a continuation of previous studies[1] and have been made with the primary aim of further elucidating certain changes occurring in the physiology of the chick embryo centering around the 12th day of incubation. At this time a secondary peak in metabolic rate occurs,[2, 3] associated with an approach of the respiratory quotient toward unity.[4, 5] Liver glycogen, first demonstrable in considerable amounts on the 7th day of development and increasing steadily until the 9th day, is reduced to a new minimum on the 12th day.[6, 7] Further, no significant change in the blood sugar level occurs at this time.[8] It is during this same period that the endocrine glands associated with carbohydrate metabolism first give evidence of active functioning. Between the 10th and 13th days of development the thyroid,[9] the medulla of the suprarenal[10] and the islets of Langerhans[11, 12] first give evidence of activity. That this onset of function in the endocrine glands was concerned in some way in these changes seemed almost certain. For this reason, extracts of the endocrine glands known to be concerned with carbohydrate metabolism in the adult mammal were injected individually into chick embryos of various ages and the resultant effects on liver glycogen and the blood sugar level were determined.

Methods. The various extracts used were injected under the chorio-allantois through the shell membrane lining the air sac. Adrenalin was diluted with distilled water immediately before injection, while thyroxin was injected in alkaline aqueous solution. Crude anterior lobe extract (Parke, Davis & Co.) and the suprarenal cortical extract, eschatin (Parke, Davis & Co.), were injected in the original concentrations. Control injections of phenol, NaOH and distilled water were made.

---

[1] Dalton, A. J., and Hanzal, R. F., *Anat. Rec.*, 1937, **70**, Suppl. No. 1, p. 33.

[2] Bohr, C., and Hasselbalch, K. A., *Skand. Arch. f. Physiol.*, 1903, **14**, 398.

[3] Murray, H. A., Jr., *J. Gen. Physiol.*, 1925, **9**, 39.

[4] Murray, H. A., Jr., *J. Gen. Physiol.*, 1926, **9**, 603.

[5] Murray, H. A., Jr., *J. Gen. Physiol.*, 1927, **10**, 337.

[6] Dalton, A. J., *Anat. Rec.*, 1937, **58**, 393.

[7] Gill, P. M., *Biochem. J.*, 1938, **32**, 1792.

[8] Zorn, C., and Dalton, A. J., *Am. J. Physiol.*, 1937, **119**, 627.

[9] Willier, B. H., *Am. J. Anat.*, 1924, **33**, 67.

[10] Lutz, B. R., and Case, M. A., *Am. J. Physiol.*, 1925, **73**, 670.

[11] Potvin, R., and Aron, M., *C. R. Soc. Biol.*, 1927, **96**, 267.

[12] Pucher, G. W., and Hanan, E. B., private communication to authors, 1937

Blood sugar determinations were made by the Jeghers and Myers' modification[13] of Folin's micro method.[14] Roughly quantitative estimates of liver glycogen were made from histological sections stained with Best's carmine. Tissues were fixed in absolute alcohol and embedded in celloidin. Blood for the blood sugar determinations was obtained by inserting a glass cannula into one of the larger vitelline veins. The embryos used were of the White Leghorn strain.

Results. The control injections mentioned above had no demonstrable effects on liver glocogen. Table I summarizes the results obtained after the injection of adrenalin and thyroxin. Adrenalin injected in approximately physiological concentrations (0.1 cc of

TABLE I.
Liver Glycogen—Adrenalin and Thyroxin Injections. 8-day Embryos.

Subst. inj.	Amt injected in 0.1 cc	Time, hr	No. of embryos	Liver glycogen
Contr.	—	—	15	12+ 3+—
Adren.	1/100,000	2	9	8+ 1+—
"	1/50,000	2	9	6+ 3+—
"	1/4,000	2	3	2+— 1 trace
"	1/2,000	2	3	1+— 2 trace
Thyr.	1/800 mg	24	3	2+ 1+—
"	1/400 "	24	3	2+ 1+—
"	1/160 "	24	3	1+ 2+—
"	1/400 "	48	10	2+ 4+— 4—
"	1/625 "	72	2	2—
"	1/800 "	72	7	7—
"	1/1000 mg	72	8	3+— 5—

TABLE II.
Liver Glycogen and Blood Sugar—Eschatin, Anterior Pituitary and Insulin Injections. 8-day Embryos.

Subst. inj.	Amt. inj. 0.1 cc	Time, hrs	No. of embryos	Blood sugar Range	Mg% Avg	Liver glyc. %+
Contr.	—	—	11	94.0-137.5	112.2	100.0
Esch.		8-16	14	94.0-154.0	128.6	7.7
Ant. Pit.		6-16	25	105.0-196.0	145.2	100.0
Insulin	4U	4	3		102.3	"
"	"	8	2		76.1	"
"	"	12	3		86.8	"
"	"	16	3		67.7	"
"	"	20	3		62.2	"
"	"	24	3		62.8	"

---

[13] Jeghers, N. J., and Myers, V. C., *J. Lab. and Clin. Med.*, 1930, **15**, 982

[14] Folin, O., *J. Biol. Chem.*, 1928, **77**, 421.

1:100,000 and 1:50,000) induces no detectable changes in the liver glycogen of 8-day embryos, although the higher concentrations of 1:4,000 and 1:2,000 do cause some glycolysis. With these high concentrations necrosis was evident. Relatively large amounts of thyroxin allowed to act for 24 hours have no effect on liver glycogen, while one gamma of thyroxin induces considerable glycolysis if allowed to act for 72 hours.

Table II summarizes the results dealing with the action of eschatin, crude anterior lobe extract and insulin on both the blood sugar level and liver glycogen of 8-day embryos. The results indicate that eschatin induces a distinct rise in the blood sugar level and a decrease in liver glycogen. The study of sections indicates some decrease in skeletal muscle glycogen also with no perceptible change in cardiac muscle or yolk sac glycogen. The injection of the anterior pituitary extract causes a marked rise in the blood sugar level together with an increase in skeletal muscle and liver glycogen. There was no obvious change in cardiac muscle or yolk sac glycogen in these cases. During the first 16 hours after the injection of insulin an almost straight-line fall in the blood sugar level occurred with an apparently associated increase in liver glycogen. The livers of embryos exposed to the action of insulin for 8 hours or more consistently showed amounts of glycogen well above the normal. It should be mentioned that, while the injection of insulin in the amounts used is invariably fatal at this time, embryos receiving either anterior pituitary extract or eschatin frequently survive and hatch.

In a previous communication[1] we concluded from the study of a smaller series that no change in liver glycogen occurred after anterior pituitary injections. The study of this larger series indicates that a definite increase in liver glycogen occurs as well as a rise in the blood sugar level.

Experiments were made to test the possibility that the low glycogen stores present in the livers of 12-day embryos might be related to the small amounts of free sugar available at this time.[15] Table III summarizes these results. It shows that raising the blood sugar above the

TABLE III.
Liver Glycogen and Blood Sugar—Glucose and Anterior Pituitary Injections. 12-day Embryos.

Subst. inj.	Amt inj.	No. of embryos	Blood sugar Range	Mg% Avg	Liver glyc. %+
Contr.	—	26	94.3-129.0	112.6	37.7
Gluc.	300 mg	21	93.0-165.0	121.2	29.4
Ant. Pit.	0.4 cc	(30)	168.0-175.0	172.0	86.2

---

[15] Needham, J., *Brit. J. Exp. Biol.*, 1926, **5**, 6.

normal level by the injection of glucose in 100 mg amounts on the 10th, 11th and 12th days does not increase the percentage of livers containing glycogen. The injection of anterior pituitary extract, on the other hand, increases liver glycogen and causes a rise in the blood sugar level just as it does on the 8th day of development. It should be mentioned that, although 30 livers were examined for glycogen in this group, blood sugar determinations were made in only 4 cases. However, these 4 had blood sugar levels above any found in either the controls or in the glucose group.

A series of 12 embryos not included in Table III were injected with 4 units of insulin on the 11th day. Examination of the livers from these embryos sacrificed on the 12th day showed that 9 contained considerable amounts of glycogen, while 3 contained only traces. This suggests that insulin is also capable of increasing the glycogen content of the livers of 12-day embryos. No blood sugar determinations were made on this group.

Summary. 1. Thyroxin and suprarenal cortical extract induce glycogenolysis of the glycogen normally present in the livers of 8-day embryos. 2. The injection of adrenalin in approximately physiological concentration appears to have no effect on liver glycogen. 3. Increased liver and skeletal muscle glycogen and a rise in the blood sugar level have been noted after the injection of crude anterior pituitary extract, suggesting a stimulation of gluconeogenesis. 4. Liver glycogen is increased in 8-day embryos by the injection of insulin and this increase is to be correlated with a rapid fall in the blood sugar level. 5. Injection of glucose fails to interrupt the normal disappearance of glycogen from the livers of 12-day embryos. 6. Both insulin and anterior pituitary extract cause an increase in the liver glycogen of 12-day embryos. The anterior pituitary extract also causes a rise in the blood sugar level at this time.

11653 P

Effects of Acute Dietary Zinc Deficiency in the Rat.*

HARRY G. DAY AND E. V. MCCOLLUM

From the Department of Biochemistry, School of Hygiene and Public Health, The Johns Hopkins University, Baltimore, Maryland.

By means of a diet furnishing about 7 μg of Zn per rat daily, studies at the University of Wisconsin[1-4] have convincingly demonstrated the indispensability of Zn in the nutrition of rats, although the Zn-deficient diet permitted the experimental rats to gain about 8 g per rat weekly during the 10 week period on the diet.[4] The findings have invalidated the conclusion from our laboratory[5] several years ago that the element probably is not a dietary essential. We have recently prepared a diet furnishing not more than 2 to 4 μg of Zn per rat daily. Young rats restricted to it quickly developed extreme degrees of deficiency. Because the studies have been temporarily interrupted, owing to the transfer of one of us (H.G.D.) to another laboratory, we have decided to make a preliminary report on the work at this time.

Diet. The diet was: casein hydrolysate (tryptic)[†] 15.00, egg white (cooked) 3.00, sucrose 66.29, salts 5.71, butter fat 10.00, and Oleum Percomorphum 50%, 2 drops per 100 g diet (approximately 2800 vitamin A units and 400 vitamin D units).

Each rat was given a daily supplement containing the equivalent of 6.4 g liver, 40 μg of thiamin and 1.0 mg of choline. The liver concentrate,[‡] dissolved in 6 volumes of water, was centrifuged. The supernatant solution was transferred to a Pyrex glass separatory funnel and the pH was adjusted to about 6. The solution was extracted repeatedly with dithizone (diphenyl thiocarbazone) dissolved in CCl_4. When no more Zn could be removed Zn-free dilute HCl was added until the pH was about 4. The excess of dithizone was removed with redistilled CCl_4. Traces of CCl_4 were removed by

---

[*] Aided by a grant from the Rockefeller Foundation.

[1] Todd, W. R., Elvehjem, C. A., and Hart, E. B., *Am. J. Physiol.*, 1934, **107**, 146.

[2] Stirn, F. E., Elvehjem, C. A., and Hart, E. B., *J. Biol. Chem.*, 1935, **109**, 347.

[3] Hove, E., Elvehjem, C. A., and Hart, E. B., *Am. J. Physiol.*, 1937, **119**, 768.

[4] Hove, E., Elvehjem, C. A., and Hart, E. B., *Am. J. Physiol.*, 1938, **124**, 750.

[5] Newell, J. M., and McCollum, E. V., *J. Nutrition*, 1933, **6**, 289.

[†] Obtained from Mead Johnson & Co., Evansville, Indiana, through the courtesy of Dr. Warren M. Cox.

[‡] Obtained from the Lederle Laboratories, Pearl River, N. Y., through the courtesy of Dr. Guy W. Clark.

vacuum. The thiamin and choline were then added to the purified liver solution.

To purify the casein hydrolysate 225 g of the dry powder were stirred into 1700 ml of water. After saturation had occurred the mixture was centrifuged. The supernatant solution was adjusted to pH 6-7 with redistilled NH_4OH and extracted with dithizone as in the case of the liver preparation. The purified solution was evaporated to dryness *in vacuo,* using a Pyrex all-glass still, and dried over Drierite.

Water was added to commercial sucrose to make a 50% solution. After adjusting the pH with NH_4OH, the solution was extracted with dithizone as in the case of liver. It was reduced to a viscous syrup by *in vacuo* distillation and the sugar was precipitated by the addition of redistilled ethyl alcohol. It was washed several times with alcohol and dried at 60° C.

Egg white was prepared by drying the heat-coagulated white of fresh eggs at 60° C.

Butter fat was melted and washed 4 or 5 times with equal volumes of hot redistilled water.

The salt mixture was composed of $Na_2CO_3.H_2O$ 9.5, $CaHPO_4.2H_2O$ 21.5, KC1 15.2, $MgSO_4.7H_2O$ 9.8, $Fe_2(SO_4)_3$ 0.95, $CuSO_4.5H_2O$ 0.10, and $MnSO_4.3H_2O$ 0.05. The $Na_2CO_3.H_2O$, KCl and $MgSO_4.7H_2O$ were purified by extracting the concentrated aqueous solutions of each with dithizone in $CC1_4$. The purified solutions were concentrated and the salts precipitated by the addition of alcohol. The $CaHPO_4.2H_2O$ was prepared by dithizone extraction of a neutralized $CaC1_2.6H_2O$ solution and combining it with a K_2HPO_4 solution similarly extracted. The other salts were purified by recrystallization of the C.P. products.

Experimental and Results. Fifteen 24-26-day-old rats from 3 different litters were placed on the diet and supplements. Seven (controls) were given 0.66 mg $ZnSO_4.7H_2O$ (0.15 mg Zn) in 0.2 ml of H_2O per rat daily. All the rats were kept on monel metal screens in Pyrex glass jars. The diet was given *ad libitum.* Water was redistilled in Pyrex glass. Two times each week all rats were given a small supplement of salts of I_2, Cd, Mo, As and F in aqueous solution.

The control rats developed normally and gave every evidence that the diet, when supplemented with Zn, was adequate. Two of the Zn-deficient rats died, one after 33 days on the diet and the other after 61 days. During 11 weeks on the diet the Zn-deficient rats gained an average of only 4 g per rat weekly and the controls averaged 16 g. Growth became greatly retarded in the Zn-deficient rats after the first 2 or 3 weeks. There was marked eczema in 2 cases. Some alopecia occurred in a few of the rats. Alkaline phosphatase[6] in the blood serum was markedly reduced but it was not affected in bone and kidney. The concentration of bone ash was decreased. In the experimental rats the average value was 55.8% and in the controls it was 62.2. There appeared to be some hemoconcentration. Carbonic anhydrase[7,8] of blood was slightly raised, but the enzyme activity per unit of red cells was unchanged. This is of special interest in relation to the finding that the purified enzyme contains 0.32% Zn.[9] The average concentration of Zn in the femurs of Zn-deficient rats was 94.7 μg per g of ash and in the controls it was 236.6 μg per g of ash. The method of Caughey, Holland and Ritchie[10] was used to estimate Zn. Detailed histological studies of the tissues will be published subsequently.

11654

Influence of H Ion Concentration on Depressant Effect of Nicotine Solutions on Ciliated Epithelium.

J. H. WEATHERBY. (Introduced by H. B. Haag.)

From the Department of Pharmacology, Medical College of Virginia, Richmond.

Ellisor and Richardson[1] report a considerable difference between the toxicity of nicotine sulfate and the free alkaloid for gold fish. While they did not actually measure the ratio between these toxicities, they were able to derive it from calculations based on their own data, and found the base to be about 6 times more toxic than the salt. They also observed that the base penetrates into gold fish from 5 to 7.5 times more rapidly than the salt. In view of these observations it seemed desirable to ascertain whether or not a similar difference is to be observed in the action of nicotine and its salt on a relatively simple physiological system such as the ciliated epithelium. This tissue was selected because it may be obtained readily from the esophagus of the frog; and ciliary activity may be considered a fair index of the physiological state of the tissue.

---

[6] Wiese, A. C., Johnson, B. C., Elvehjem, C. A., Hart, E. B., and Halpin, J. G., *J. Biol. Chem.*, 1939, **127**, 411.

[7] Roughton, F. J. W., *Physiol. Rev.*, 1935, **15**, 241.

[8] Lambie, C. G., *Edinburgh Med. J.*, 1938, **45**, 373.

[9] Keilin, D., and Mann, T., *Nature*, 1939, **144**, 442.

[10] Caughey, R. A., Holland, E. B., and Ritchie, W. S., *J. Assn. Off. Agric. Chem.*, 1938, **21**, 204.

[1] Ellisor, L. O., and Richardson, C. H., *J. Cell. and Comp. Physiol.*, 1938, **11**, 377.

Experimental Methods. Ciliated epithelium used in these experiments was obtained from *Rana pipiens.* The nicotine was specially prepared, and known to be of a high degree of purity. The salt (acetate) was prepared by the addition of glacial acetic acid until the desired pH was obtained. The ratio between free base and salt in solutions of known pH was obtained by application of the formula $pH = pK + \log. base/salt$, in which pK was found experimentally to be 8.07. At pH 8.07 approximately 50% of the total nicotine is present as the free base, whereas at pH 6.5 only about 2% exists as base, the remainder being in the form of the salt. Solutions at higher or lower pH values cannot be used because of the H-ion effects on the tissue.

The experimental methods are similar in many respects to those described by Richardson[2] and by Perrine, Throndson, and Tainter;[3] although certain modifications were found advisable. The method is as follows: A section of the esophagus 5 to 8 mm in length was removed and divided lengthwise into 2 strips. Each strip was pinned to a piece of cork so that it lay relatively smoothly without being under undue tension. Mucus was removed as completely as possible, usually by gentle mopping with a small pledget of cotton. The tissue was moistened with frog Ringer's solution of approximately the same pH as the nicotine solution being investigated. Ciliary activity was ascertained by measuring the length of time required for a small piece of cork to be swept across a certain distance on the surface of the tissue. For these observations a small lens having a magnification of about 4X was used; and to the under side of the lens were attached 2 parallel hairs about 2 mm apart. Time required for the cork to pass between the 2 hairs was measured with a stop-watch. Two groups of 5 observations each were made on each strip to indicate normal or control conditions. The piece of cork bearing the tissue was then placed, tissue down, into the solution being investigated, and allowed to remain for 5 minutes. It was then removed, and another series of 5 observations made. The average of these 5 observations was then compared with the average of the 10 observations previously made to indicate the change in ciliary activity. Observations on several hundreds of strips, both control and experimental, indicate that changes of less than 25% are of doubtful significance. In most in-

---

[2] Richardson, A. P., *J. Pharmacol. and Exp. Therap.*, 1937, **59**, 101.

[3] Perrine, R. L., Throndson, A. H., and Tainter, M. L., *J. Dental Research*, 1939, **18**, 81.

TABLE I.
Influence of pH on Depression of Ciliary Activity Caused by Solutions of Nicotine

pH	Concentration %	Control time, sec	Experimental time, sec	$\frac{\text{Control}}{\text{Experimental}}$	Remarks
6.5	1.0	4.43	5.02	0.882	
”	2.0	4.27	8.62	0.495	
”	4.0	5.27	12.92	0.408	
”	6.0	4.91			All sections stopped
8.0	0.5	4.36	5.76	0.757	
”	1.0	4.69	13.02	0.360	
8.10	1.5	4.45	15.60	0.285	5 of 10 sections stopped
8.05	2.0	4.39			All sections stopped

stances 10 strips were used for each concentration of nicotine at each of the hydrogen ion concentrations.

Results. In Table 1 are shown the effects of nicotine solutions of different concentrations at pH 6.5 and pH 8.0-8.1. As stated above, at pH 8.07 approximately 50% of the total nicotine is present as the free base, whereas at pH 6.5 only about 2% exists as the base, the remainder being in the form of salt. Thus, a threefold increase in total nicotine was necessary to produce the same physiological effect (stoppage of ciliary motion) at pH 6.5 as at pH 8.05. At the same time there was a decrease in free nicotine content from 50% to 2%. At pH 10 more than 99% of the nicotine is present as the free base, but such a solution could not be used because of the OH-ion effect. Therefore, it was not possible to make a direct comparison between solutions containing practically no free base and others containing practically no free salt. However, since an increase in free base from 2% to 50% increases toxicity threefold, a further increase in free base to 100% might reasonably be expected to cause a proportionate increase in strength of action to about six times that of the salt, a ratio in satisfactory agreement with that reported by Ellisor and Richardson.[1]

Conclusion. A change in the physiological activity of a nicotine solution accompanies a change in pH of the solution when the phenomenon of transport across membranes is involved. An increase in pH causes an increase in the proportion of free base present, and, as pointed out by Ellisor and Richardson,[1] an increase in the rate at which nicotine passes through membranes. This is believed to account for the fact that a solution containing equal parts of nicotine acetate and the free base is about three times more effective in inhibiting ciliary activity than a solution containing about 98% of the acetate.

11655 P

Influence of Methylcholanthrene on Age Incidence of Leukemia in Several Strains of Mice.*

ARTHUR KIRSCHBAUM,[†] LEONELL C. STRONG AND W. U. GARDNER

From the Department of Anatomy, Yale University School of Medicine, New Haven, Conn.

It has been reported that a large number of mice of the dba strain developed leukemia when painted on the skin with methylcholanthrene or 9:10 dimethyl-1:2 benzanthracene dissolved in benzene.[1] There are other reports concerning the effect of carcinogens on the induction of leukemia in mice.[2]

The question arises as to whether carcinogens actually induce leukemia in mice of non-leukemic strains or whether the administration of these agents accelerates the appearance and perhaps increases the incidence of this disease only in mice showing a tendency towards its spontaneous development. Mice of the inbred F strain which shows a high incidence of spontaneous leukemia[3] were painted with methylcholanthrene twice weekly according to the technic of Morton and Mider.[1] Mice of the C3H strain received similar treatment. Other non-leukemic strains (NH, CHI, CBAN and C57) were painted once a week. Painting with methylcholanthrene was begun at an average age of 30 days. The essential findings are summarized in Table I.

Most of the painted F mice which did not develop leukemia were sacrificed or died because of the development of large ulcerating skin tumors which appeared between the ages of 100 and 200 days. It was not possible with this technic, therefore, to determine whether the incidence of leukemia in the F strain can be increased by administration of methylcholanthrene. In the C3H strain 3 lymphoid neoplasms appeared before 300 days of age among 94 mice painted. Of 280

---

* This investigation was aided by grants from the Fluid Research Fund of Yale University School of Medicine, the Jane Coffin Childs Memorial Fund for Medical Research and the Anna Fuller Fund.

[†] Finney-Howell Research Foundation Fellow, 1939-40.

[1] Morton, J. J., and Mider, G. B., *Science*, 1938, **87**, 327; Mider, G. B., and Morton, J. J., *Am. J. Cancer*, 1939, **37**, 355; Law, L. W., and Lewisohn, M., PROC. SOC. EXP. BIOL. AND MED., 1940, **43**, 143.

[2] Parsons, L. D., *J. Path. and Bact.*, 1935, **40**, 45; Burrows, H., and Cook, J. W., *Am. J. Cancer*, 1936, **30**, 75; Furth, J., and Furth, O. B., *Am. J. Cancer*, 1938, **34**, 169; Lewis, M. R., *Am. J. Cancer*, 1938, **34**, 399; Brues, A. M., and Marble, B. B., *Am. J. Cancer*, 1939, **37**, 45.

[3] Kirschbaum, A., and Strong, L. C., *Am. J. Cancer*, 1939, **37**, 400.

TABLE I.
Age Incidence of Leukemia in Untreated Mice and Mice Painted with Methylcholanthrene (F strain—leukemic; C3H strain—non-leukemic).

Age in days	No. of Leukemias	Type of disease			No. of Leukemias	Type of disease		
		ML*	LL†	MedLym‡		ML*	LL†	MedLym‡
	Untreated F mice—125 animals				F mice painted with MC—83 animals			
0-100	0	0	0	0	1	1	0	0
100-200	4	0	1	3	24	11	10	3
200-300	9	0	2	7	4	0	2	2
300-400	18	4	8	6				
400-500	16	3	12	1				
500-600	17	7	10	0				
600-700	5	3	1	1				
	—	—	—	—	—	—	—	—
Totals	69	17	34	18	29	12	12	5
	Untreated C3H mice—280 animals				C3H mice painted with MC—94 animals			
0-100	0	0	0	0	0	0	0	0
100-200	0	0	0	0	2	0	0	2
200-300	0	0	0	0	1	0	1	0
300-400	0	0	0	0				
400-500	1	0	1	0				
500-600	2	0	2	0				
600-700	0	0	0	0				
	—	—	—	—	—	—	—	—
Totals	3	0	3	0	3	0	1	2

*Myelogenous leukemia.
†Lymphatic leukemia.
‡Mediastinal lymphosarcoma—very large lymphoid tumor in the mediastinum, usually relatively little systemic disease.

controls no leukemias or lymphosarcomata were observed in this age group (Table I). No leukemias appeared in 90 mice of the CHI, NH, C57 and CBAN strains which were painted once a week for from 3-14 months.

The age at which leukemia appeared and the type of leukemia developed in F mice were influenced by painting with methylcholanthrene. In untreated mice of the F strain only 4 of 69 cases of spontaneous leukemia appeared before 200 days of age. Of the 29 leukemias observed following painting with methylcholanthrene, however, 25 appeared before 200 days of age. Myelogenous leukemia appears in untreated F mice only after 300 days of age (17 cases—Table 1).[‡] In mice painted with methylcholanthrene, on the other hand, a case of myelogenous leukemia appeared at 97 days of age after 73 days treatment, a total of 12 cases appearing before 200 days of age. (Two of these were transplanted, myelogenous leukemia developing in the inoculated mice.) Most untreated F mice which develop lymphoid neoplastic disease before 300 days of age exhibit massive mediastinal tumors. The proportion of such tumors was not increased in mice of this age group when treated with methylcholanthrene. These experiments demonstrated that mice of the F strain, when painted with methylcholanthrene will develop between 97 and 200 days of age a type of leukemia characteristic for mice of an older age group (300-700 days).

[‡] Myelogenous leukemia in the F strain has been described.[3]

Summary. Leukemia appeared at an earlier age than in controls when mice of the F strain were painted twice weekly with methylcholanthrene. Myelogenous leukemia, which does not occur in untreated F mice before 300 days of age, appeared as early as 97 days after birth in treated mice. Only 3 cases of leukemia occurred in 184 mice of non-leukemic strains treated in a similar manner. The effectiveness of methylcholanthrene in influencing the appearance of leukemia in young mice depended on the genetic susceptibility of mice to the disease.

11656

Microestimation of Leucine, Isoleucine, and Valine.

RICHARD J. BLOCK, DIANA BOLLING AND A. A. KONDRITZER.†

From the Department of Biochemistry, New York State Psychiatric Institute and Hospital, New York City.

Arginine, histidine, lysine, cystine, methionine, tyrosine, tryptophane, phenylalanine, glycine, alanine, and threonine can all be determined by available micro methods[1, 2] in only 2 to 4 g of protein, but until recently methods were not available for the estimation of 3 of the essential amino acids: leucine, isoleucine, and valine in small quantities of protein. Last year Fromageot and Heitz[3] described a procedure for the determination of leucine and valine, involving deamination to the corresponding hydroxy acids with nitrous acid, oxidation to acetone with chromate, and measurement of the acetone colorimetrically after reaction with salicylaldehyde. Leucine and valine were estimated in the presence of each other by carrying out the oxidation with chromate on 2 aliquots under conditions such that the proportionate yield of acetone from the 2 amino acids differed considerably. We were unsuccessful in obtaining consistent results with this procedure. In the present study we have made extensive changes in and simplified the procedure of Fromageot and Heitz and in addition have found conditions which permit the determination of isoleucine by measuring the ethylmethylketone formed in the oxidation of the corresponding hydroxy acid. All 3 amino acids can be determined in as little as 100 mg of protein for no one of the other amino acids recognized as occurring in proteins yields acetone or ethylmethylketone under these conditions.

---

† Research Associate in Biochemistry, Neuro-Psychiatric Institute, Hartford Retreat, Hartford, Conn.

[1] Block, R. J., *The Determination of the Amino Acids*, Burgess Publishing Company, Minneapolis, Minn., Rev. Ed., 1938.

[2] Block, R. J., and Bolling, D., *J. Biol. Chem.*, 1939, **130**, 365.

[3] Fromageot, C., and Heitz, P., *Enzymologia*, 1939, **6**, 258.

Hydrolysis and Deamination. The protein is hydrolyzed with 10 volumes of 8 N sulfuric acid and the excess acid is removed with barium hydroxide. The precipitate of barium sulfate is centrifuged and thoroughly washed with hot water. The filtrate and washings are reduced *in vacuo* to 50 cc. Amino acid solutions prepared by other methods of hydrolysis are suitable provided the hydrolyzing agent does not yield compounds which would interfere in later steps. Five or 10 cc aliquots of the solution are removed and the amino acids are deaminated with an excess of sodium nitrite, a few drops of sulfuric acid being added to keep the solution acid to Congo paper. The excess nitrous acid is destroyed by warming the solution on the steam bath from 10 to 15 minutes.

*Oxidation.** Instead of oxidizing with chromate under 2 different conditions (under pressure and with continuous distillation)[3] we employ 2 different oxidizing agents: chromate and permanganate.

A. 2 g of $K_2Cr_2O_7$ are dissolved in 20 cc of 5 N sodium acetate buffer at pH 4.7 in a 3 x 20 cm test tube into which a stream of air is passed in order to aspirate the acetone and ethylmethylketone into 2 water traps connected in series and kept in an ice bath. The oxidation mixture is boiled and the solution of hydroxy acids is introduced from a burette at such a rate that the volume remains constant. Time: 30 minutes. The aeration and boiling are continued until the volume of the oxidizing solution is reduced to 10 cc (30 minutes). The aqueous solutions in the traps are combined and diluted to volume. Under these conditions valine yields 47% and leucine 6% of the theoretical amount of acetone. Isoleucine yields 52% of the theoretical amount of ethylmethylketone.

B. 2 g of $KMnO_4$ are dissolved in 20 cc of M phosphate buffer at pH 6.8 and the oxidation is carried out in the same way as described above. Under these conditions valine yields 50% and leucine 36% of the theoretical amount of acetone. Isoleucine yields 52% of the theoretical amount of ethylmethylketone.

---

* During the course of this investigation the following oxidizing agents have been tried alone or in combination, $Ce(HSO_4)_4$; $Ce\ (HSO_4)_4\text{-}I_2$; $(CH_3COO)_4Pb$; PbO_2; $K_2Cr_2O_7\text{-}CH_3COOH$; $K_2Cr_2O_7\text{-}NaOH$; $K_2Cr_2O_7\text{-}H_2SO_4$; $KMnO_4\text{-}KOH$; $KMnO_4\text{-}H_2SO_4$; $KMnO_4\text{-}H_3PO_4$; MnO_2; $MnO_2\text{-}H_3PO_4$; KIO_4; KIO_3; HIO_4; Ag_2O; Caro's reagent; $K_2S_2O_8\text{-}AgNO_3\text{-}H_2SO_4$; H_2O_2; $H_2O_2\text{-}FeSO_4$; and others.

Estimation of Leucine and Valine. The amounts of acetone present in the chromate and permanganate distillates are determined by precipitation with Denigés' reagent or colorimetrically by condensation with salicylaldehyde.[4] Under these conditions ethylmethylketone fails to give a color. Some of the data obtained are given in Table I. The amounts of valine and leucine are calculated from the following equations: Valine $= 4.85$ Acetone$_{K_2Cr_2O_7}$-0.81 Acetone$_{KMnO_4}$; Leucine $= 7.54$ (Acetone$_{KMnO_4}$-Acetone$_{K_2Cr_2O_7}$).

TABLE I.
Oxidation of Leucine, Isoleucine, and Valine by $K_2Cr_2O_7$ and $KMnO_4$.

	$K_2Cr_2O_7$ %	$KMnO_4$ %
Leucine	6.7,5.9,5.9,6.3,6.2	36,34,32,39,34,41
Isoleucine	50,50,49,53,51,55,56,53	54,52,52,47,53,53,52,53
Valine	49,48,44,49,49,45	53,48,50,53,47

Estimation of Isoleucine. A. Acetone is precipitated with Denigés' reagent from a portion of the aqueous solution obtained from either the chromate or permanganate oxidation of the hydroxy acids. The filtrate is diluted to a volume such that aliquots of 1 to 2 cc will contain from 0.005 to 0.05 mg of ethylmethylketone. Such aliquots are diluted to 8 cc, 4 cc of absolute alcohol are added, and after cooling to 0°, 4 cc of concentrated sulfuric acid are introduced. The solution is mixed and 2 cc of 10% alcoholic salicylaldehyde are added. The tube is stoppered and, after mixing is placed in a water bath at 50° over night. The color is read using a 520 mu filter. A calibration curve covering the range of 0.005 to 0.05 mg of ethylmethylketone is prepared. Isoleucine=3.5 x ethylmethylketone.

B. Although acetone gives color with salicylaldehyde in the presence of sulfuric acid, the amount is much less than that yielded by ethylmethylketone. Therefore, it is possible to determine ethylmethylketone directly without removal of acetone by means of calibration curves. Both procedures should be employed.

The recovery of leucine, isoleucine, and valine, when a mixture of these 3 amino acids and glycine was oxidized in the proportions 24, 6, 8, and 250 respectively, was leucine+2%, valine+7%, and isoleucine+6% for chromate oxidation and +1% for permanganate oxidation above the amounts expected from the formulae. It should be stressed that standard conditions must be developed to ensure the same percentage oxidation each time.

---

[4] Block, R. J., and Bolling, D., *The Determination of the Amino Acids,* Burgess Publishing Company, Minneapolis, Minn., Second Ed., 1940.

Up to the present 35 proteins have been analyzed for leucine, isoleucine, and valine (cf. [4], Table II for some of the results).

11657

Rapid Absorption of Substances Injected into the Bone Marrow.

L. M. TOCANTINS

From the Division of Hematology, Jefferson Medical College and Hospital, Philadelphia.

Substances injected into the marrow cavity of the tibia of the rabbit and of the sternum of man appear to find their way immediately into the general circulation. That material so administered is taken up and utilized as rapidly as if it had been injected intravenously has been demonstrated in the following ways:

1. Blood replacement by intramedullary injection. A rabbit was bled of 20% of its calculated blood volume, by puncturing the heart and aspirating the blood slowly into a solution of sodium citrate. Twenty-four hours afterwards a needle with bevelled stylet was placed into the marrow cavity of the upper portion of the tibia, and blood, freshly removed from another animal, was introduced in an amount equivalent to that withdrawn the preceding day. The injection was allowed to proceed at the rate of about 5-7 cc per minute. Slight twitching of the leg after the injection started was the only disturbance observed in the animal. Seven animals were treated as described; 2 other animals were bled and allowed to recover spontaneously. Of the 7 treated animals 4 recovered their original (previous to the bleeding) erythrocyte and hemoglobin level within 24 hours after the intramedullary injection, 2 within 48 hours, and one died as a result of a hemopericardium. The last mentioned animal was the only fatality among all the animals that received various substances by the intramedullary route. None of the other animals showed any sign of distress during or after the experiments. In Fig. 1 is illustrated the response of the erythrocytes, hemoglobin and reticulocytes of a treated and an untreated animal.

2. Injections of glucose in experimental hypoglycemia. Four rabbits were rendered hypoglycemic by intravenous injections of a dose

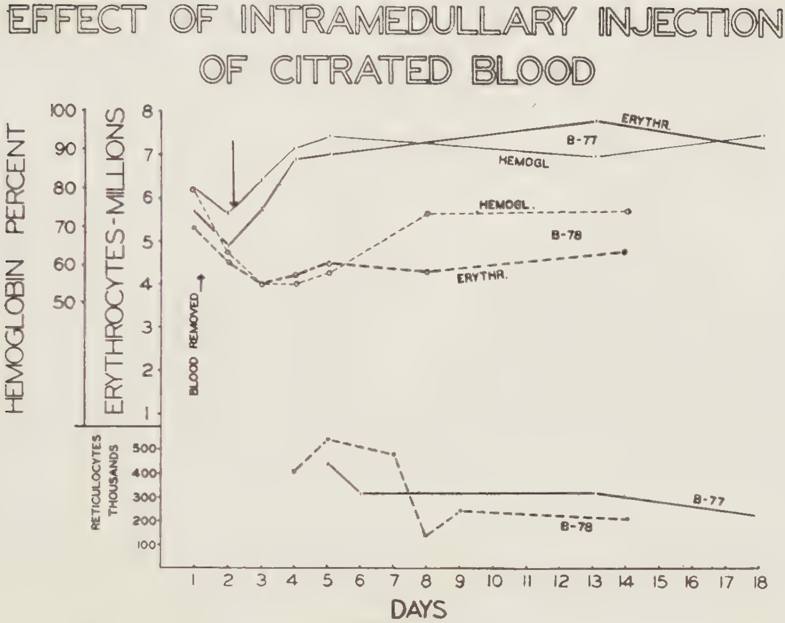


FIG. 1.

Blood was removed by heart puncture from both animals (short arrow). 24 hours later an amount of freshly collected blood equivalent to that removed was injected (long arrow) into the tibial marrow of Rabbit B-77.

of insulin equivalent to 12 units per kilogram of body weight. Immediately after the appearance of a convulsion (usually within 3 hours after the injection) a 25 or 30% solution of dextrose (2-3 g per kilo body weight) was injected into the marrow cavity of the upper portion of the tibia at the average rate of 8 cc per minute. The abdominal reflex which was always absent during the convulsive period returned immediately after the end of the injection and there were no further convulsions. All dextrose treated animals recovered from the hypoglycemic reaction. An additional animal to which the dextrose injection was not given died 37 minutes after the first convulsion.

Rabbit B-85. Weight 3.3 Kg. Fasting 24 hours.

7-25-40 11:00 A. M.: Blood sugar (Benedict's method): 102 mg per 100 ml of blood.

11:02 A.M.: 22 units of crystalline zinc insulin intravenously.

12:33 P.M.: 18 units of zinc insulin intravenously.

12:42 P.M.: 2 convulsive seizures; abdominal and corneal reflexes absent. Blood sugar: 64 mg.

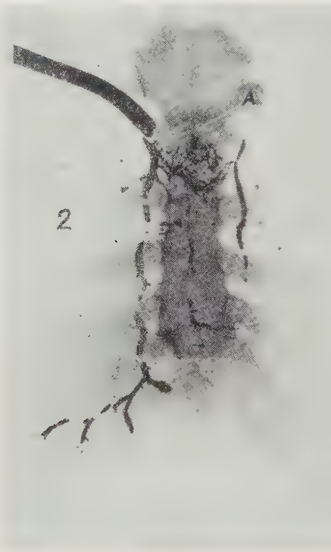
12:50-12:52 P.M.: 20 cc of 25% glucose injected into the marrow of the tibia. Abdominal and corneal reflexes present at the end of the injection. No further convulsions.

12:54 P.M.: Blood sugar: 540 mg.

1:03 P.M.: Blood sugar: 363 mg. Animal alert and active.
3:50 P.M.:Blood sugar: 78 mg. Animal fed and watered.
7-26-40 9:00 A.M. Animal well. Blood sugar (not fasting): 152 mg.

3. Rapidity of spread of dye injected in the tibial marrow. A marrow puncture needle was inserted into the upper portion of the tibia of a rabbit. A plain 20 gauge needle was introduced into the heart and 1.5 cc of blood aspirated into 0.5 cc of 1.3% sodium oxalate. The needle was left in the heart and meanwhile 1.5 cc of a 1% solution of Congo Red was injected through the tibial needle into the marrow cavity. At 10, 20 and 60 second periods following the end of the injection (duration of injection 7″) blood was removed from the heart through the indwelling needle. The specimens were then centrifuged and the color of the plasmas noted. The plasma was colored in the very first specimen, indicating that it took ten seconds or less for the material to reach the heart from the tibia. Similar results were obtained with a solution of fluorescein.

4. Injection of mercury into the marrow cavity of the tibia of the rabbit and of the sternum of man. Fig. 2 represents an X-ray photograph of a human sternum and its collateral rib fragments removed at autopsy. A needle had been inserted at point A and about 1 cc of



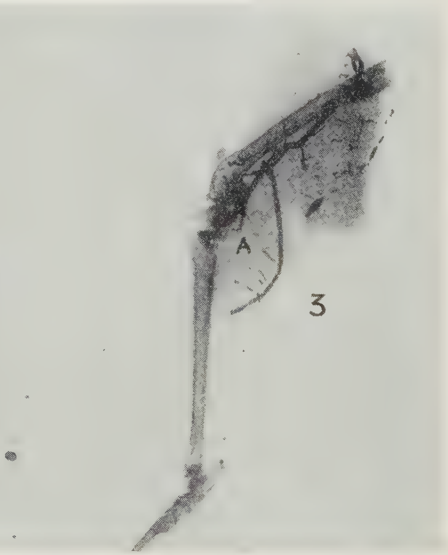


FIG. 2.
Sternum and collateral rib fragments of an adult negress. The thick line at the upper left corner is a hemostat clamping the cut end of the right internal mammary vein.

FIG. 3.
Lower extremity of a rabbit. Amputation was carried out before injection. Arrows indicate points of injections of the mercury.

mercury injected under slight pressure. Almost immediately after the beginning of the injection, mercury was running out of the severed ends of the internal mammary veins on the under surface of the sternum. The same technic was followed in an attempt to determine the path of outlet of mercury injected into the upper portion of the tibia of a rabbit (Fig. 3). Mercury so injected apparently finds its way through veins in the bone cortex into the deep femoral. Just as in the human sternum, the path of least resistance appears to be toward the venous system. In man the marrow tissue itself of the sternum, adjacent to the point of injection, was infiltrated to a limited extent, the mercury escaping chiefly into the outgoing venous channels; in the rabbit the rest of the tibia was entirely free of the injected material.

5. Gravity infusion of physiological salt solution into the sternum of man.* The sternal bodies of 3 adult men were punctured with a specially built gauge 15 needle, holding within it a gauge 18 needle with a bevelled stylet. Marrow was aspirated through the smaller gauge needle which was then removed. The lumen of the larger needle was then filled with physiological salt solution and connected with a salt solution infusion apparatus. In one man, complaining of bone pains, it was not possible to infuse any solution by this method; in the other 2, salt solution ran in by gravity readily, without discomfort, the infusion being maintained at rates varying between 5-10 cc per minute, for periods as long as 30 minutes when the experiment was discontinued.

Josefson[1] injected as much as 5 cc of liver extract into the sternal marrow cavity of patients with pernicious anemia with the object of stimulating the bone marrow directly. A transient headache, sometimes severe, accompanied by vomiting, often followed these injections. These symptoms were probably caused by the massive rapid absorption of the drug, for they may be observed also after intravenous injections of liver extract. Radio opaque substances have been injected into the sternum of living patients with the object of studying structural changes in the marrow; the method succeeded only partially, because the injected material escaped into the general circulation before adequate photographs could be obtained.[2] The medulla of bones may, therefore, offer one more route for parenteral therapy when quick absorption is desired and prevailing circumstances (poor or obliterated veins, extensive burns or mutilations) make it difficult or impossible to use the common paths.

---

* From the wards of Medical Service B, Pennsylvania Hospital.

[1] Josefson, A., *Acta Medica Scand.*, 1934, **81**, 550.

[2] Berthet, G., Benda, R., Orinstein, E., and Depitre, *Sang.*, 1940, **14**, 172.

Summary. Substances injected into the marrow cavity of the tibia of the rabbit and of the sternum of man are almost immediately absorbed into the general circulation. Blood and glucose solutions respectively, by intramedullary injection, corrected rapidly experimental anemia and hypoglycemia induced in rabbits.

11658

Formation of the Anti Egg-White-Injury Factor (Biotin) in the Rumen of the Cow.

L. W. McElroy and T. H. Jukes

From the Divisions of Animal Husbandry and Poultry Husbandry, University of California, Davis.

When raw egg white is added to a nutritionally adequate diet, characteristic skin symptoms are produced in various species. The symptoms may be prevented or cured by supplementation with a vitamin-like factor.[1] Evidence has recently been presented[2] that this "anti egg-white-injury" factor is identical with biotin.

It has recently been shown that thiamin, nicotinic acid, riboflavin, pantothenic acid, pyridoxine and vitamin K may be formed in the rumen of the cow.[3] The present experiments indicate that the anti egg-white-injury factor (biotin) is also formed under the same conditions.

In the present experiments, the characteristic symptoms of egg-white-injury were produced in chicks by feeding the following basal diet (Diet ED): Yellow cornmeal, 55 g; wheat middlings, 20; dried skim milk, 10; commercial casein, 10; ground limestone, 2; steamed bonemeal, 2; alfalfa meal, 1; NaCl, 0.5; $MnSO_4$, 0.05; fresh raw egg white, 30 cc. The wet mixture was spread in thin layers and allowed to dry at room temperature, after which 0.3 g of fish oil blend (3000-A, 400-D) was added. Chicks were placed on this diet at hatching. They developed the characteristic symptoms of the syndrome in 3 to 4 weeks. The dermatitis appeared simultaneously at the commissures of the mouth, below the lower mandible, and on the feet. Keratinization and fissuring of the feet were especially striking symptoms. This differentiates the gross symptoms from those of pantothenic acid deficiency, in which the dermatitis first appears at the commissures of the mouth only. Perosis, which in this case was not prevented by choline, also was associated with the egg-white syndrome.

---

[1] Boas, M. A., *Biochem. J.*, 1927, **21**, 712; György, P., *J. Biol. Chem.*, 1939, **131**, 733.

[2] György, P., Melville, D. B., Burk, D., and du Vigneaud, V., *Science*, 1940, **91**, 243; du Vigneaud, V., Melville, D. B., György, P., and Rose, C. S., *Science*, 1940, **92**, 62.

[3] McElroy, L. W., and Goss, H., *J. Biol. Chem.*, 1940, **133**, lxv; *J. Nutrition*, in press.

A sample of a diet for ruminants,[4] deficient in the B-complex, was fed as a supplement to diet ED. Simultaneously another group of chicks received as a supplement a sample of the dried rumen contents of a cow[3] fed the ruminant diet. Another group of chicks received a supplement of cane molasses. Other groups received respectively the basal diet ED and a supplement of pantothenic acid. The chicks were observed at frequent intervals for symptoms of "egg-white dermatitis". The results in Table I were obtained.

TABLE I.
Effect of Various Supplements on Development of the Egg-white Syndrome in Chicks. Ten chicks were used in each group. Diet ED was used as the basal diet.

Supplement in 100 g of diet	No. of birds showing dermatitis at: 24 days	26 days	Wt in g at: 20 days	26 days
None	6	7	113	141
10 g ruminant ration	6	7	86	100
10 g dried cow rumen contents	0	0	142	193
70 mg pantothenic acid	8	7	129	138
10 g cane molasses (standard control)	1	1	131	165

The data for dermatitis are given for the 24th and 26th days, since the symptoms developed most rapidly during the fourth week. The cane molasses was kindly assayed by Dr. E. E. Snell for biotin by a microbiological method. It was found to contain 2.6 μg of biotin per gram. Assuming that the anti egg-white-injury factor is identical with biotin, it appears that the dried cow-rumen contents was a slightly better source of biotin than the cane molasses. The biotin content of the ruminant diet was apparently as low as, or lower than the chick basal diet ED. Evidently the anti egg-white-injury factor (biotin) was formed in the rumen, presumably by microbial synthesis. Pantothenic acid was ineffective at a level corresponding to 50 times the requirement of the chick.

Summary. The anti egg-white-injury factor (biotin) was found to be formed in the rumen of a cow receiving a ration which was low in this factor.

---

[4] McElroy, L. W., and Goss, H., *J. Biol. Chem.*, 1939, **130**, 437.

11659 P

Low Calcium Rickets in the Guinea Pig.*

P. R. Howe, L. G. Wesson, P. E. Boyle and S. B. Wolbach
From the Forsyth Dental Infirmary, and the Medical and Dental Schools of Harvard University.

The purpose of the present investigation was to determine if rickets could be produced in the guinea pig by diets low in calcium and low in vitamin D. This is of importance since there is a widespread belief that the guinea pig can dispense with vitamin D, perhaps by synthesizing it analogously to the synthesis of vitamin C by the rat and other animals.

No report of low calcium rickets in the guinea pig has been found. Harris[1] published a roentgenograph and a photomicrograph of a rachitic guinea pig, and, in a personal communication states that a high calcium-low phosphorus diet (Steenbock and Black) was used. Emerique[2] also reported rickets in guinea pigs, produced by a high calcium-low phosphorus diet, and that a curative effect was secured by the use of vitamin D for 7 days. No reference to tooth changes in rachitic guinea pigs has been discovered, but in rachitic dogs[3] and rats[4] the changes in developing teeth include hypoplastic defects in the enamel and a wide dentinoid border in the dentin.

Diet. Twenty-one guinea pigs weighing 166-380 gm were selected and during the experimental period were kept in a shaded room. The diet was composed of a mixture of dextrin, 71%; extracted casein, 15%; dried brewers' yeast (Harris), 6%; butter fat, 5.9%; and a calcium-free salt mixture, 2.1%. The diet was furnished in the form of a thick paste. Roughage was obtained by means of strips of filter paper. In addition, each pig received daily 10 g lettuce, 50 mg ascorbic acid, and 0.05 mg carotene.

The salt mixture is practically neutral. It contains the necessary inorganic constituents, except Ca and PO_4, in such a proportion that 2.1% will give approximately the same percentage of these constituents in the synthetic diet as 3.5% of the complete salt mix-

---

* Aided by a grant from the Carnegie Corporation to the University Committee on Research in Dental Medicine.

[1] Harris, H. A., *Bone Growth in Health and Disease,* Oxford University Press, London, 1933.

[2] Emerique, L., *Compt. rend. Acad. Sci.,* 1937, **205**, 879.

[3] Mellanby, M., *Physiol. Rev.,* 1928, **8**, 545.

[4] Becks, H., and Ryder, W. B., *Arch. Pathol.,* 1931, **12**, 358.

ture previously recommended.[5] Because of the sulphur and phosphorus of the casein and yeast, the diet as a whole is somewhat acid, the acidity being equivalent to about 1% H_2SO_4. The Ca and P contents of the synthetic portion of the diet are respectively 0.028% and 0.200% by analysis. The food intake varied widely both as to the individual animal and the duration of the experimental diet. If it be assumed that 20 g of the synthetic mixture were consumed

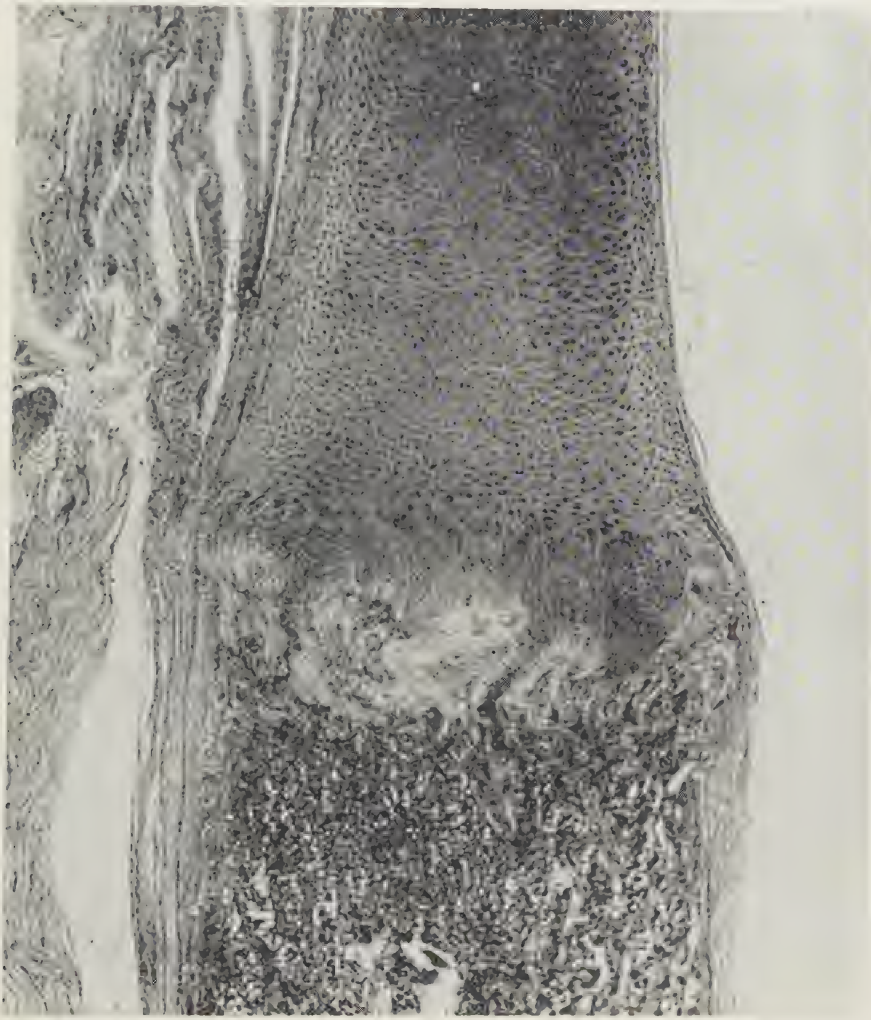


FIG. 1.

Costochondral junction of a guinea pig kept for 60 days on a low Ca, low vitamin D diet. The widening of the zone of proliferative cartilage, irregular vascular penetration and masses of osteoid tissue are characteristic of rickets. × 45.

---

[5] Wesson, L. G., *Science,* 1932, **75**, 339.

daily along with the 10 g portion of lettuce (1.7 mg Ca and 4.0 mg P[6]), there was a daily intake of approximately 7.3 mg Ca and 44 mg P, giving a P/Ca ratio of 6.0.

Findings. Nine animals survived from 44 to 60 days on the experimental diet, and are the subject of the present report. The body weight usually remained stationary after an initial loss until the final week or 2 weeks when, in all instances, it decreased. Widening and irregularity of the zone of cartilage proliferation at the epiphyseal lines of the long bones was most marked in the younger

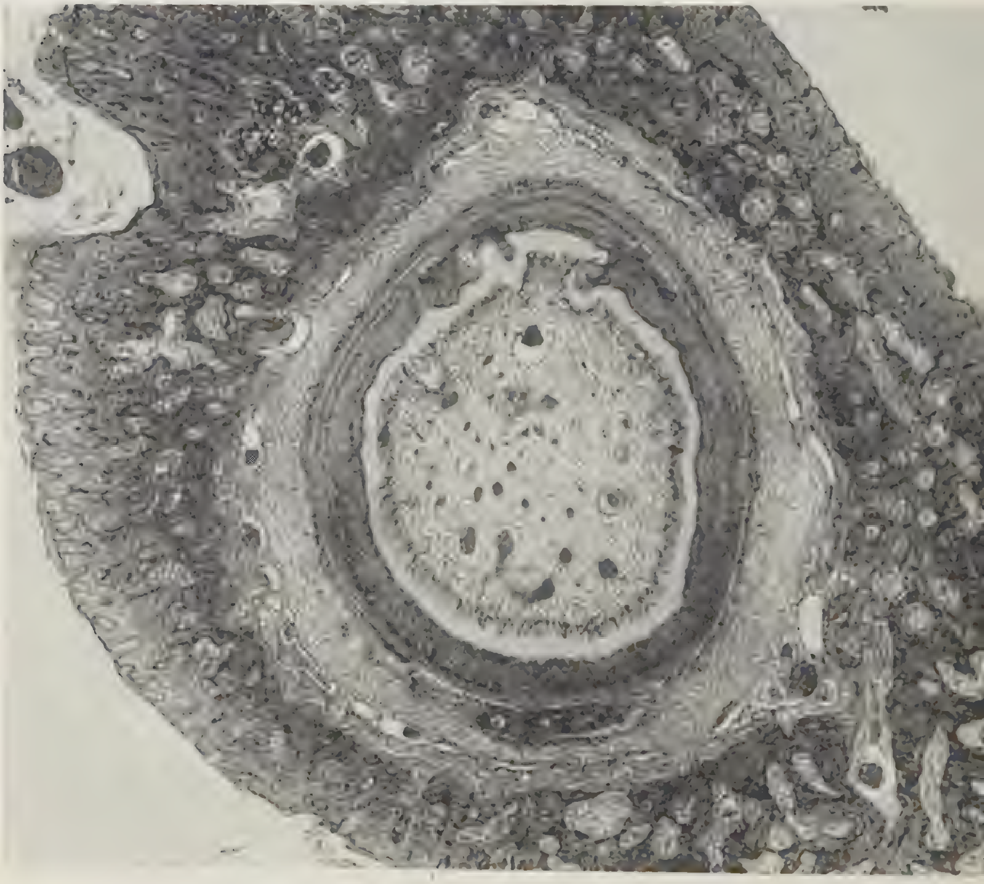


FIG. 2.

Cross section of the lower incisor tooth of the same animal cut at the level of the mental foramen. A mass of uncalcified enamel matrix may be seen on the lower mid part of the outer surface of the tooth. Almost no other enamel was present in this region. The irregularity of the calcification of the dentin and the wide uncalcified inner margin (dentinoid) are also characteristic of rickets. × 45.

---

[6] Sherman, H. C., *Chemistry of Food and Nutrition*, Macmillan, New York, 1937, p. 592.

animals. The number of cells in the cartilage columns was increased, and long tongues of cartilage and osteoid extended into the bony shafts. These changes were equally pronounced in the ribs (Fig. 1). In the older animals arrest of growth occurred and histological signs of rickets were thus not observed in the bones.

The teeth exhibited an extreme degree of enamel hypoplasia (Fig. 2). Areas of complete failure of enamel deposition occurred. In other areas, irregular masses of enamel matrix were found which appeared to be Ca-free since, in undecalcified sections, they did not stain with $AgNO_3$ or alizarin red S. The dentin also showed irregular calcification and a dentinoid margin 2 to 5 times normal in width. The rate of dentin deposition, as measured by intravital injections of alizarin red S,[7] was approximately half normal. Distortion of the shape of the tooth often occurred. These rachitic changes in the teeth were not affected by the arrest of skeletal growth as were the changes in the bones.

Summary. Typical rachitic changes in bones and teeth have been produced in young guinea pigs by a diet low in calcium and in vitamin D. In older animals the changes in the bones were obscured by arrested skeletal growth, but those in the teeth were constant.

11660

Convenient Electroencephalographic Electrode.

CHESTER W. DARROW. (Introduced by E. Gellhorn.)

*From the Institute of Juvenile Research and Department of Physiology, University of Illinois, Chicago.**

To make contact with the scalp for electroencephalographic recording the practice has frequently been to cut the hair in small areas and fasten electrodes in place with collodion. The collodion and the electrodes were subsequently removed with ether. The attaching of a dozen or more such electrodes for an electroencephalographic exploration is laborious, the cosmetic effect is not too favorable, and the patient may not be enthusiastic.

A more expeditious procedure for placing of electrodes on hair covered areas is possible with an electrode which makes use of the hair itself to hold the electrode. Two simple electrodes are here described. The first, illustrated in Fig. 1, consists of a ring of metal ½″ in diameter, 3/16″ high, with a wire lead attached, and a slot 3/32″ deep in the top into which fits a wire "hairpin" of No. 20 spring wire bent double like a cotter pin or a "bobby pin". After massage of an area of scalp with electrode paste a wisp of hair from the area is passed through the metal ring and inserted in the slot of the wire hairpin. The wisp of hair is wound up, windlass fashion, on the hairpin until definite tension holds the pin against the top of the electrode ring and pushes the electrode ring against the scalp. The doubled wire pin is then permitted to slip edgewise into the slots in the top of the electrode ring. This prevents the pin from turning or the hair from unwinding, and the electrode ring is held snugly in place. The ring may be filled with electrode paste, insuring contact and providing assurance against drying of the electrode. Corrugations or comblike teeth may be milled on the lower edge of the electrode ring so that stray hairs beneath the ring may not prevent contact with the scalp.

---

[7] Boyle, P. E., Bessey, O. A., and Howe, P. R., *Arch. Pathol.*, 1940, **30**, 90.

* Series C, No. 313.

A still more expeditious though not more efficient method of attachment is provided by the electrode in Fig. 2 where a "jack-knife" arrangement is soldered in the top of the electrode ring. A wisp of hair is laid across the slot or scabbard portion of the jack-knife and the blade closed on top of it. If the distal end of the wisp of hair is held during the closure of the blade, tension will be developed at the scalp end of the wisp of hair and hold the electrode tightly in place. As in the previously described arrangement electrode jelly is placed in the electrode ring.

Little or no discomfort is occasioned by a very considerable pull on the wisp of hair holding the electrodes. There is apparently no disturbance of the encephalogram by local stimulation. Removal of the electrodes requires only a moment. They may be suitably plated to prevent corrosion by the electrode paste or jelly.

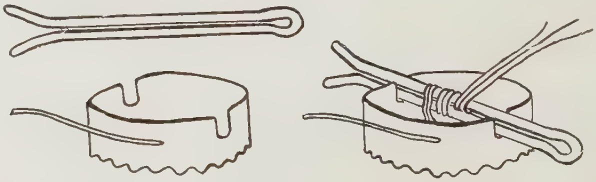


FIG. 1.

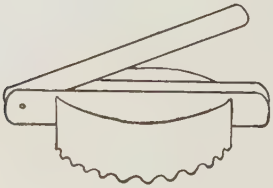


FIG. 2.

11661

Adsorbing Charcoals in Chick Diets.

H. J. ALMQUIST AND D. ZANDER.

From the Division of Poultry Husbandry, University of California College of Agriculture, Berkeley.

It has long been a common practice to add charcoal to poultry feeds, under the impression that charcoal improves the health and reduces the mortality rate of chickens. Acceptable evidence for these alleged attributes has been lacking. Charcoal is claimed to act by adsorbing undesirable products from the digestive tract, for example, one manufacturer of poultry charcoal lists the following substances as removed: carbon dioxide, ammonia, bacteria, pigments, poisons and enzymes.

Charcoal is known to cause a bleaching of poultry flesh and of egg yolk by reducing the deposition of carotenoid pigments from the diet. This bleaching effect suggests the possibility that carotene and other vitamins in the diet might also be removed or inactivated by charcoal. For example, former experience in this laboratory showed that vitamin K is adsorbed strongly on charcoal and that it cannot later be removed without much destruction. The vitamin K-deficient chicken fed vitamin K adsorbed on charcoal seems unable to remove and utilize the vitamin.[1]

A diet was formulated to provide optimum amounts of vitamins A, K, G (riboflavin) and the chick gizzard factor, without the considerable margins of safety that exist in most good commercial chick mashes. This diet was found capable of promoting normal growth and health of chicks.

The diet was composed of the following:

Sardine fish meal	20.0
Dried brewer's yeast	2.0
Dried skim milk	2.0
Dried alfalfa	0.5
Sardine oil, fortified*	0.05
Calcium carbonate	0.5
Wheat bran	10.0
Ground polished rice	62.0
Granite grit	2.0
Salt	0.05

In the first experiment, day-old chicks were fed the basal diet with and without 2% of an adsorbing charcoal. Results are given in Table I.

---

*This oil contained at least 400 chick units of vitamin D and 3000 U.S.P. units of vitamin A per gram.

[1] Unpublished data of the senior author.

TABLE I.
Results of Experiment I.

Diet	Basal	Basal + 2% charcoal 1
No. of chicks	20	20
Mortality	2	3
Avg wt at 34 days	294.6 g	181.3 g
Cases of vitamin A deficiency	0	15
Avg blood clotting time	2.4 min	over 30 min
Condition of gizzard linings	normal	all eroded

Vitamin A deficiency occurring in the charcoal-fed group was diagnosed from the typical paralysis and incoördination, keratinization of the nictitating membrane, and poor growth. All cases recovered after daily oral administration of vitamin A in fish oil. Severe vitamin K deficiency was identified by the presence of subcutaneous hemorrhages in some birds and a prolonged blood clotting time in all birds fed charcoal. Gizzard factor deficiency was in evidence in the case of the severely eroded gizzard linings.[2] Three distinct deficiencies had, therefore, been produced by the addition of an adsorbing charcoal to the diet.

In Experiment II a second sample of adsorbing charcoal was tested. Results are given in Table II.

In this experiment, vitamin A deficiency was not in evidence. There was in the charcoal group, however, a slight prolongation of the blood clotting time, indicating some loss of vitamin K, and a moderate occurrence of gizzard erosion, indicating some loss of the gizzard factor. Another deficiency became manifest in the development of "curled-toe paralysis" a symptom of vitamin G (riboflavin) deficiency. Four of the 6 cases developed in the charcoal group were treated with synthetic riboflavin orally and all 4 cases were promptly cured. The lack of evident vitamin A deficiency and the appearance of vitamin G deficiency in Experiment II as compared to Experiment I may have been due to differences in the reserve stores of these vitamins in the different lots of chicks.

TABLE II.
Results of Experiment II.

Diet	Basal	Basal + 2% charcoal 2
No. of chicks	20	20
Mortality	0	0
Avg wt at 34 days	310.5 g	314.2 g
Cases of vitamin A deficiency	0	0
Avg blood clotting time	4.2 min	8.4 min
Condition of gizzard lining	normal	several eroded
Cases of "curled-toe" paralysis	0	6

---

[2] Almquist, H. J., and Mecchi, E., *J. Biol. Chem.*, 1938, **126**, 407.

TABLE III.
Results of Experiment V.

Diet	Practical mash	Practical mash + 2% charcoal[2]
No. of chicks	35	35
Mortality	0	2
Avg wt at 10 days	67.6 g	67.6 g
" " " 52 "	521.6	545.8
" gain per unit of feed	0.328	0.332

A repetition of Experiment II gave similar results. In Experiment IV, both samples of charcoal were fed simultaneously to separate groups of chicks from the same lot. Results were again similar to those obtained with each charcoal sample separately, while chicks in the same lot receiving no charcoal maintained the best growth and normal health. In a fifth experiment, charcoal 2 was added at a level of 2% to a practical chick rearing mash fed to a pen of carefully selected chicks. A similar pen received only the mash. This practical mash was richly supplied with all the factors in question and it is not surprising that no outward deficiency symptoms were noted. Results are given in Table III.

The results of this last test are typical of attempts to demonstrate any beneficial effect from feeding charcoal to chicks. Differences in growth are slight and usually not significant. Results from other tests (Exp. IV) have yielded slight differences in the opposite direction; and it seems impossible to confirm these differences one way or the other. This same difficulty has been encountered by others in investigations of charcoal in chick rations.[3]

In conclusion, it may be stated that a chick diet adequate for normal growth and health was rendered, in effect, deficient in vitamins A, G, K and the gizzard factor by the addition of an adsorbing charcoal. These findings point to the possibility of some measure of this effect in practical rations supplemented with charcoal if the mash does not contain more than adequate levels of these factors, or if the mash consumption is reduced by heavy scratch grain feeding, or if the birds have unlimited access to charcoal. The adsorbing power of charcoals in poultry rations is not necessarily a completely beneficial property.

---

[3] Private communication from Professor G. F. Heuser concerning the thesis of George C. Hodgson, Cornell University, 1938.

11662

Convulsive Action of Triphenyl Phosphite.*

Robert B. Aird, Waldo E. Cohn and Sidney Weiss. (Introduced by D. M. Greenberg.)

From the Departments of Surgery and Biochemistry, University of California Medical School, San Francisco and Berkeley, California.

Triphenyl phosphite has been used as a convulsive agent in experimental epilepsy.[1] Because it is relatively easy to prepare this compound synthetically[2] and to render it radioactive by synthesizing it from radioactive phosphorus,† it seemed feasible and desirable in our studies on epilepsy to combine the function of tracer substance with that of convulsant. Since it was eventually demonstrated that triphenyl phosphite hydrolyzes and the convulsant effects were due to the phenol fraction which is known to act at the cord level,[3] its use was abandoned. In the course of these studies, however, certain interesting observations were made in connection with the absorption, hydrolysis, toxicity and distribution of triphenyl phosphite. The results seem worthy of brief report, inasmuch as this compound belongs to the same series as triorthocresyl phosphite, which produces a combined system degeneration of the spinal cord[4] and is related to triorthocresyl phosphate, the contaminant responsible for the degenerative changes in cases of Jamaica ginger paralysis.[5]

The toxic doses of triphenyl phosphite as reported by Smith, *et al.*,[4] were confirmed in rats and cats. In dogs 2 doses of as little as 0.5 cc per kilo of body weight, given one week apart, produced the

---

* Aided by grant from Child Neurology Research (Friedsam Foundation). We wish to acknowledge the helpful advice and criticism of Professor David M. Greenberg.

[1] Cobb, S., Cohen, M. E., and Ney, J., *J. Nerv. and Ment. Dis.*, 1937, **85**, 438; *Arch. Neurol. and Psychiat.*, 1938, **40**, 1156.

[2] Noack, E., *Ann. der Chemie*, 1883, **218**, 96; Gottlieb, H. B., *J. Am. Chem. Soc.*, 1932, **54**, 748.

† We are indebted to Professor E. O. Lawrence and members of the Radiation Laboratory for the radioactive phosphorus used in this study. It was prepared by bombardment of phosphorus with deuterons accelerated in the cyclotron.

[3] Baglioni, S., *Centralbl. für Physiologie*, 1909, **14**, 97; *Z. f. allgem. Physiol.*, 1909, **9**, 1.

[4] Smith, M. I., Lillie, R. D., Elvove, E., and Stohlman, E. F., *J. Pharm. and Exp. Therap.*, 1933, **49**, 78.

[5] Smith, M. I., Elvove, E., and Frazier, W. H., *U. S. Pub. Health Rep.*, 1930, **45**, 2509.

typical ataxia and weakness in the hind legs within 2 weeks. The early convulsive reaction was found to depend upon the purity of the preparation. A commercial preparation proved most toxic and 2 synthetic preparations, in one of which a high degree of freedom from contamination with phenol was achieved, indicated that the convulsive effects could be closely correlated with the degree of phenol contamination of the preparation.

Smith and his coworkers[4] have adduced evidence purporting to show that the esters of phosphorous acid were absorbed very slowly from the site of injection. Because this might be correlated with the delayed onset of the neurological changes it is a point of considerable importance. In our own studies methylene blue, erythrosin-B, and eosin were found to concentrate at the site of inflammatory reactions, such as are produced by the injection of the esters of phosphorous acid, and are not necessarily related to the specific presence of the phosphites. Only 8% of the radioactive triphenyl phosphite (P^{32}) was regained unabsorbed one hour and 45 minutes after its injection into the peritoneal cavity.

Quantitative determinations of free phenol[6] and radioactive phosphorus (as triphenyl phosphite and hydrolyzed phosphite) were made on the cerebrospinal fluid, blood, motor cortex, diencephalon, and remainder of the cerebral hemispheres of the brains of 8 cats 1½ hours after the intraperitoneal injection of 0.3 cc of radioactive triphenyl phosphite per kilo of body weight. The radioactive preparations varied from 7,500 U‡ to 15,000 U in strength of radioactivity. The results are shown in Table I. One other experiment, in which 0.5 cc of radioactive triphenyl phosphite (30,000 U) per kilo of body weight was injected intraperitoneally, gave results comparable with those shown in Table I and in addition showed 0.011% P^{32} per g of fluid in the cerebrospinal fluid, 0.0032% P^{32} per g of tissue in the muscles and steadily rising values of P^{32} in the blood up to the conclusion of the experiment at one hour and 45 minutes after the injection of the radioactive triphenyl phosphite.

Although the variations of the determinations are wide, it is evident in all instances that only a small amount of radioactive phosphorus (triphenyl phosphite or hydrolyzed phosphite) reached the central nervous system in 1½ hours and at this same time there was a considerable excess of free phenol beyond that normally found in these tissues. If the injected triphenyl phosphite is assumed to be completely hydrolyzed and the phosphorous acid fraction absorbed

---

[6] Folin, O., and Ciocalten, V., *J. Biochem.*, 1927, **73**, 627.

‡ U—arbitrary unit of radioactivity, approximately equal to 10^{-3} μC.

TABLE I.

Determinations of Radioactive Phosphorus (P^{32})* and Free Phenol (⬡OH)† 1½ Hours After the Intraperitoneal Injection of 0.3 cc/kg Body Weight of Radioactive Triphenyl Phosphite.

Cat	Dose of P^{32} U	C.S.F. ⬡OH†	Blood		Motor Cortex		Diencephalon		Brain Residue	
			P^{32}*	⬡OH†	P^{32}*	⬡OH†	P^{32}*	⬡OH†	P^{32}*	⬡OH†
1	15,000	1.84	2.5	7.3	0.3	10.6	0.17	10.5	1.7	4.2
2	15,000	1.13	0.77	4.2	0.3	7.3	0.7	8.6	0.14	0.83
3	10,000	0.95	0.35	2.96	10.0	—	0.4	13.5	0.25	—
4	15,000	1.83	0.89	8.4	2.3	—	2.0	—	0.053	—
5	15,000	2.3‡	0.27	5.9	2.0	5.65	0.23	6.25	2.4	6.04
6	7,500	0.83	0.29	3.2	0.8	12.4	0.1+	5.3	0.12	6.5
7	10,000	0.74	0.70	4.7	7.3	4.96	1.4	5.02	0.60	5.3
8	15,000	0.98	0.19	6.0	0.15	11.0	6.5	5.1	0.30	6.05
Avg		1.32	0.74	5.33	2.89	8.65	1.44	7.75	0.695	4.82
Ratio	⬡OH/P^{32}		7.2		3.0		5.38		6.93	

*% of dose injected per 100 g of tissue.
†Mg%.
‡Contaminated with blood.

and distributed to a degree comparable to the phenol fraction, the value of the ratio of free phenol, in mg %, to radioactive phosphorus, in % per hundred g, averaged over all 8 cats (average weight 2.75 kg) would be 9. A slightly higher value would be obtained if the free phenol factor is not corrected for the free phenol normally present in the tissues. Inasmuch as the detoxification of phenol (oxidation, conjugation, and elimination) occurs relatively rapidly in the body, the free ⬡OH/P^{32} ratios of the average observed values should be lower approximations of this theoretical value. This would be true only in case hydrolysis were essentially complete and the absorption and distribution of the hydrolyzed fractions occurred to a comparable degree within the 1½-hour time limit of these experiments. Since the values found in the case of the bloods and brain residues did approximate this theoretical ratio, it is clear that triphenyl phosphite was hydrolyzed readily *in vivo*, that the hydrolyzed fractions were widely distributed, and that this affords an adequate explanation for the fact that the phenol fraction was present in the central nervous system in great excess and in amounts sufficient[4] to account for the early, toxic manifestations which involved the central nervous system. The lower ratios for the diencephalon and motor cortex were due to the relatively higher concentrations of P^{32} found in these tissues, which suggests a selective and preferential absorption of the phosphorous acid fraction by the gray matter of the central nervous system.

The precise distribution in spinal cord, medulla, and cerebellum of the radioactive phosphorus and the concentrations necessary to produce the late, degenerative changes in the central nervous system were not determined. Since, however, detoxification of the phenol fraction is known to occur rapidly and does not cause degenerative changes in the central nervous system, the late degenerative changes associated with triphenyl phosphite may be presumed to be caused by relatively small concentrations of phosphorus acid acting over long periods. The preferential absorption of the phosphorous acid fraction by the gray matter is of interest in this connection.

11663

Effect of Pantothenic Acid on the Nutritional Achromotrichia.

KLAUS UNNA AND W. L. SAMPSON. (Introduced by H. Molitor.)
From the Merck Institute for Therapeutic Research, Rahway, N. J.

Greying of the fur was first observed by Morgan, Cook and Davison[1] and Lunde and Kringstad[2] in black rats maintained on diets deficient in the vitamin B complex. Addition of preparations containing "filtrate factor",[3] of liver extracts containing pantothenic acid[4] or of brewers' yeast[2] have been reported to prevent the development of greying or to restore the black pigmentation of the hair.

In our laboratory, greying of the hair was obtained in black and piebald rats on a diet consisting of casein, vitamin free, 18%; sucrose, 67%; butter fat, 9%; salt mixture, 4%; cod liver oil, 2%, and supplemented with thiamin, riboflavin, nicotinamide and vitamin B_6. On this diet greying of the fur developed in approximately 80% of the animals within 4 weeks. At this time, the animals reached stationary weights and showed, in addition to the grey symmetrical patterns of the fur, signs characteristic of pantothenic acid deficiency in rats,[5] namely, thinning of the fur, generalized scaly dermatitis, inflammation of the nasal mucosa, blood-caked whiskers, and hemorrhages in various organs, particularly in the adrenal cortex. The addition of graded doses of pantothenic acid (calcium pantothenate, Merck) to the diet demonstrated that a daily supplement of 80 or 100 μg of calcium pantothenate, *e. g.*, doses representing the daily requirement for pantothenic acid in rats,[5] prevented, besides the above mentioned deficiency symptoms, the greying of the fur, whereas daily doses of 5, 10, or 20 μg were insufficient to prevent the greying, and 40 μg gave inconsistent results.

---

[1] Morgan, A. F., Cook, B. B., and Davison, H. G., *J. Nutr.*, 1938, **15**, 27.

[2] Lunde, G., and Kringstad, H., *Z. f. physiol. Chem.*, 1939, **257**, 201.

[3] Morgan, A. F., and Simms, H. D., *J. Nutr.*, 1940, **19**, 233.

[4] György, P., Poling, C. E., and Subbarow, Y., *J. Biol. Chem.*, 1940, **132**, 789.

[5] Unna, K., *J. Nutr.*, 1940, in press.

Comparable results were obtained with various concentrates from rice bran and liver. Although the addition of these concentrates caused better growth responses than pantothenic acid alone, their efficacy in preventing the greying of the hair was found to parallel closely their content of pantothenic acid as measured by the bacteriological assay.[6] This was shown by the addition of 10% rice bran, containing 30 μg of pantothenic acid per gram, to the diet. Assuming a food consumption of 5 to 10 g per rat, these animals received 15 to 30 μg of pantothenic acid daily. Greying of the fur as obtained under these conditions was comparable with that of the rats receiving 20 μg of calcium pantothenate daily. However, the growth rate of the rats receiving the rice bran was markedly superior, the difference being probably due to the presence of unknown growth factors in rice bran. Furthermore, the addition of 50 μg of calcium pantothenate to the diet containing 10% rice bran prevented the greying of the fur.

Calcium pantothenate, administered daily in doses of 100 μg to rats which had been maintained on the deficient diet for 6 to 10 weeks, produced a striking growth stimulation. The external deficiency symptoms and the grey color of the hair showed a somewhat delayed response. Simultaneously with an improvement of the general condition of the fur and skin, black hair started to grow. The grey patterns disappeared gradually within 3 to 5 weeks. By this time, the smoothness and the luster of the coat were restored together with the black pigmentation. The persistence of some scattered grey hairs in some of the animals gave them a "pepper and salt" appearance. With rice bran or liver concentrates similar curative effects were obtained on grey haired rats. Since the administration of large amounts of liver extracts sometimes also restored the black color of the fur only to the "pepper and salt" stage, it is felt that this might be due rather to a genetic factor in our strain than to a failure of pantothenic acid.

The results demonstrate the relation between the greying of the hair and the dietary deficiency in pantothenic acid. Furthermore, they give evidence of both the preventive and curative effect of calcium pantothenate as compared with that of concentrates of rice bran or liver. These experiments are not concerned with the possibility of preventing greying of hair by substances other than vitamins. Studies of this nature on black rats of a pure bred strain are in progress.

---

[6] Snell, E. E., Strong, F. M., and Peterson, W. H., *Biochem. J.*, 1937, **31**, 1789.

11664 P

Toxicity of Pantothenic Acid.

Klaus Unna and Joseph Greslin. (Introduced by H. Molitor.)
From the Merck Institute for Therapeutic Research, Rahway, N. J.

Pantothenic acid, first discovered as a growth factor for yeast,[1] has been established as a member of the vitamin B complex.[2] The vitamin was recently identified[3, 4] as N-(α,γ-dihydroxy-β, β-dimethylbutyryl)-β'-alanine and has been synthesized.[5]

In continuation of our studies of the toxicology of the vitamins of the B complex,[6, 7] the following investigation of the toxicity of pantothenic acid was carried out on animals maintained on completely adequate diets. Synthetic dextrorotatory calcium pantothenate was used in all experiments. Calcium pantothenate is readily water-soluble and almost neutral in reaction, the pH of a 10% solution being approximately 8.

Local effects of calcium pantothenate were studied by instillation into the eye and by subcutaneous injection. Instillation of 0.5 cc of a 10% solution into the conjunctival sac of 3 rabbits did not produce any irritation. Likewise no irritation, inflammation or abscess formation was observed in 4 rabbits following the subcutaneous injection of 1.0 cc of a 10% solution. The infiltration of the subcutaneous tissues subsided about as rapidly as following a control injection of 10 cc of saline.

Acute toxicity was studied in mice, rats, dogs, and monkeys. In mice and rats the L.D. 50 was determined following oral, subcu-

---

[1] Williams, R. J., Lyman, C. M., Goodyear, G. H., Truesdail, J. H., and Holaday, D., *J. Am. Chem. Soc.*, 1933, **55**, 2912.

[2] Williams, R. J., *Science*, 1939, **89**, 486.

[3] Williams, R. J., and Major, R. T., *Science*, 1940, **91**, 246.

[4] Stiller, E. T., Keresztesy, J. C., and Finkelstein, J., *J. Am. Chem. Soc.*, 1940, **62**, 1779.

[5] Stiller, E. T., Harris, S. A., Finkelstein, J., Keresztesy, J. C., and Folkers, K., *J. Am. Chem. Soc.*, 1940, **62**, 1785.

[6] Unna, K., *J. Pharm. and Exp. Therap.*, 1939, **65**, 95.

[7] Unna, K., and Antopol, W., Proc. Soc. Exp. Biol. and Med., 1940, **43**, 116.

TABLE I.
Acute Toxicity of Calcium Pantothenate L.D. 50 (g per kg body wt).

	Oral	Subcutan.	Intraper.	Intrav.
Mice	10.0	2.7	0.92	0.91
Rats	No deaths at 10.0	3.4	0.82	—

taneous, intraperitoneal and intravenous injection, 10 animals being used for each dose level. Table I summarizes the results obtained from a total of 180 mice and 110 rats.

All 10 rats dosed with 10 g of calcium pantothenate per kg, when administered by mouth, survived without showing toxic symptoms. Lethal doses in mice and rats produced prostration and respiratory failure. Death occurred within one hour following intravenous or intraperitoneal injection and within 2 hours following oral or subcutaneous administration. No late deaths were observed.

A dose of 1 g of calcium pantothenate per kg was fed to each of 5 dogs and to 1 monkey. No toxic symptoms were observed in these animals. After 2 weeks the monkey and one dog were sacrificed; gross examination of the organs failed to show any pathologic changes.

Chronic toxicity was studied in rats, dogs, and monkeys. Two groups of 20 young rats, 10 males and 10 females, were fed 50 mg and 200 mg respectively of calcium pantothenate per rat per day over a period of 120 days. The animals developed normally, their growth rate did not differ significantly from that of the control group.

A group of 6 adult dogs was fed 50 mg of calcium pantothenate daily over a period of 100 days, and 4 monkeys of 2.5 to 4 kg body weight received 1 g of calcium pantothenate by mouth daily over the same period of time. Both dogs and monkeys failed to show any toxic symptoms, and their weights remained constant.

Our findings, which will be extended later by histological examinations, demonstrate that calcium pantothenate is, like other members of the vitamin B complex, a substance of extremely low toxicity. Excessively large doses (0.8 g to 10 g per kg, depending on the mode of administration) were found to produce death due to respiratory failure in mice and rats. Prolonged feeding of sublethal doses failed to produce toxic manifestations, indicating that an excess of pantothenic acid is either rapidly destroyed or excreted. Studies on this problem are now in progress.

11665

Effect of Oxidized Bile Salts on Gall Bladder Bile.

B. P. Phibbs, H. S. Wigodsky and A. C. Ivy.

From the Department of Physiology and Pharmacology, Northwestern University Medical School, Chicago.

The salts of the oxidized bile acids are known to be markedly choloretic. Whether or not this choloresis increases the flow of bile through the gall bladder or modifies some of the physical characteristics of gall bladder bile is not known. The present study was undertaken to determine the effect of a chloresis induced by oxidized bile salts on some of the physical characteristics of gall bladder bile in dogs.

Intravenous or oral administration of bile salts at the same time as, or following, the administration of tetraiodophenolphthalein has been demonstrated to cause an increase in the size of the X-ray picture of the gall bladder.[1] This evidence is indirect, but indicates that the choloresis following the administration of bile salts results in an increased flow of bile into the gall bladder.

Beck, *et al.*,[2] have determined the effect of an extract of ox bile on the pH and concentration of gall bladder bile in dogs. They concluded that, because of the high correlation between total solids and pH, concentrated bile will possess a greater hydrogen-ion concentration than those which are more diluted.

Methods. Two groups of dogs, each containing 28 animals, were used in this experiment. All of the dogs received one-half can of prepared dog food 3 times a day for 3 days. The treated animals received in addition 0.75 g of a mixture of ketocholanic acids (Ketochol) by mouth with each feeding. On the fourth day, 12 hours after the last feeding, the abdominal wall was incised, under ether anesthesia, the cystic duct was clamped and divided, and the gall bladder removed intact.

The pH of the gall bladder bile was determined by means of the glass electrode. The bile was withdrawn into the electrode chamber immediately after removal of the gall bladder from the dog. Specific gravity was determined by the falling drop method and in some instances checked by the gravimetric method, both methods yielding almost identical results. The percentage of solids was determined

---

[1] Taterka, H., *Med. Wilt.*, 1930, **4**, 1722; Impallomani, R., *Radiologica Medica*, 1938, **25**, 583; Jankelson, I. R., and Altman, W. S., *Radiology*, 1933, **21**, 448.

[2] Beck, F. F., Krantz, J. C., Jr., Feldman, M., and Carr, C. J., Proc. Soc. Exp. Biol. and Med., 1937, **37**, 357.

TABLE I.

No. animals			pH	Sp.Gr.*	% solids	Cholesterol†
Controls	28	Avg	6.6	1.0408	22.86	85.3
		Min.	5.9	1.0314	17.18	54.5
		Max.	8.4	1.0506	29.27	163.0
Treated	28	Avg	6.6	1.0398	22.31	88.8
		Min.	5.8	1.0290	14.92	63.0
		Max.	8.0	1.0459	29.33	125.0

*Average of 38 samples in each group.
† " " 19 " " " "

by drying known quantities of bile in a vacuum oven for 72 hours at 100°C and 20 lb of vacuum. The samples were allowed to cool in a desiccator and weighed on a "Chainomatic" balance containing a calcium chloride tube in the balance case. Cholesterol determinations were made on mixed samples, according to the method of Elman and Taussig.[3]

Included in the specific gravity results are the findings in 10 treated and 10 untreated gall bladder biles upon which no other determinations were made.

The averaged results and ranges are shown in Table I. There are no significant differences between the control and treated animals. The quantity of bile salts given was sufficient to insure a maximum choloresis well within the time limit of the experiment.[4] No conclusions as to the flow of bile through the gall bladder can be drawn from these data. It can be assumed that if there is an increased flow of bile through the gall bladder due to the choloresis caused by the administration of ketocholanic acids, the gall bladder is able to concentrate the "choloretic bile" as well as normal bile. We have no way of knowing whether the concentrated choloretic bile is discharged and the gall bladder refilled, or whether the bile remains in the gall bladder until a meal is taken as in the normal animal.

The normal values for pH and percent of solids obtained in this experiment agree well with those of Beck, *et al.*[2]

Conclusions. The choloresis produced in dogs by the administration of oxidized ketocholanic acids does not affect the pH, specific gravity, percentage of solids, or the cholesterol content of the gall bladder bile.

---

[3] Elman, R., and Taussig, J. B., *J. Lab. and Clin. Med.*, 1931, **17**, 274.

[4] Berman, A. L., Snapp, E., Ivy, A. C., Atkinson, A. J., and Hough, V. S., *Am. J. Digest. Dis.*, 1940, **7**, 333.

11666

Involution of Thymus During Pregnancy in Young Mice.

EDWARD C. PERSIKE, JR. (Introduced by C. H. Danforth.)

From the Department of Anatomy, Stanford University.

By injecting Antuitrin S, Butcher and Persike[1] caused an arrest in growth of the thymi of young albino rats of both sexes. This effect was apparently mediated through the gonads, since the injections were ineffective in arresting the growth of the thymi of gonadectomized animals. Pregnancy urine was used by Klein[2] to obtain an involution of the thymus in guinea pigs.

Jolly and Lieure[3] reported that with 4 exceptions, the thymi of 20 pregnant guinea pigs weighed one-third to one-half as much as did those of their non-pregnant controls. Since 20% of the animals used by Jolly and Lieure apparently showed no reduction in the size of their thymi during pregnancy, and since it was thought to be worth while to test the effect of pregnancy on the thymus of the mouse, another experimental animal, the following experiments were performed.

Only healthy litter-mate virgin mice were used, each group being kept in a separate cage in which there was an excess of food and water at all times, since it has been shown that malnutrition may be an important factor in causing thymic involution.[4, 5] Each group consisted of 2 litter-mate female mice with identical body weights at the beginning of the experiment. One was utilized as a control, and the other was mated as soon as it became sexually mature, thus avoiding as much of the normal age-involution as possible. Both mice in each of the 20 groups were killed on the day the pregnant mouse gave birth to young. Body weights and the number of young were recorded. At autopsy, the thymi were dissected free from the surrounding tissues and weighed on a quantitative balance, weights of postpartum thymi being recorded as percentages of their respective controls (Table I). The majority of the specimens were examined for histological changes.

In the postpartum mice there was found to be a thinning out of the thymocytes in both cortex and medulla, resulting in a less distinct differentiation between these two zones, and accompanied by an invasion and replacement of the normal thymic substance by connective and adipose tissues. Similar changes have been observed by other investigators, who obtained an involution of the thymus by administration of Antuitrin S,[1] pregnancy urine,[2] and androgenic and estrogenic substances.[6, 7]

---

[1] Butcher, E., and Persike, E., *Endocrinology*, 1938, **23**, 501.

[2] Klein, F., *Klin. Wchnschr.*, 1936, **15**, 371.

[3] Jolly, J., and Lieure, C., *Compt. rend. Soc. de Biol.*, 1930, **114**, 451.

[4] Hammar, J., *Z. f. mikr.-anat. Forschung*, 1926, **6**, 670.

[5] Capper, A., and Schless, R., *J. Pediatrics*, 1934, **4**, 573.

The data indicate that the thymi did undergo involution during pregnancy. Assuming that the thymic weights in the 2 members of each group were the same at the beginning of the experiment, this involution varied between 45% and 80%, except in one pair, where the thymus of the postpartum mouse was heavier than that of its control. These data on the mouse accord with those of Jolly and Lieure[3] for the guinea pigs, indicating that pregnancy does cause definite involution of the thymus. The greater consistency of results in these experiments over those of Jolly and Lieure, is probably due to the use of litter-mates of the same body weight at the time of mating. There appears to be no correlation between the number of young born and the degree of thymic involution. Although each mated mouse and its control were initially of the same body weight, the majority of the postpartum mice weighed more than their controls at the time of autopsy.

TABLE I.
Data Taken at Autopsy on Day of Littering by the Test Animals.

Body wt, g			Thymus wt, mg		
Control	Postpartum	No. of young	Control	Postpartum	% Decrease
24	27	2	40	9	78
21	25	2	32	18	44
21	21	3	37	18	51
20	24	4	20	24	0
21	26	4	50	21	58
29	32	4	47	22	53
19	25	4	39	12	69
30	34	5	38	19	50
22	27	5	38	10	74
29	27	5	55	19	66
26	28	6	36	21	42
23	26	7	30	11	63
26	28	7	35	18	49
20	29	7	37	6	84
21	24	7	37	15	60
20	25	8	32	12	63
21	18	8	37	6	84
19	23	8	33	13	61
23	25	8	31	13	58
37	33	10	58	20	66

---

[6] Chiodi, H., *Rev. Soc. Argent. de Biol.*, 1938, **14**, 309.

[7] Persike, E., *Am. J. Physiol.*, 1940, **130**, 384.

Summary. Pregnancy caused the thymi of young mice to undergo involution, both grossly and microscopically. With one exception out of 20, the degree of thymic involution varied between 45% and 80% as measured by gland weights. At parturition, the postpartum mice generally weighed more than their litter-mate controls.

11667

Permeability of Red Blood Cells to Sulfathiazole.*

JOHN G. REINHOLD, LEON SCHWARTZ, HARRISON F. FLIPPIN, AND SHLOMITH BETHLAHMY. (Introduced by D. Wright Wilson.)
From the Committee for the Study of Pneumonia, Philadelphia General Hospital.

Differences in permeability of cells to sulfanilamide and its derivatives promise to be of some importance in the investigation and application of these compounds. Sulfanilamide has been shown to penetrate red blood cells and tissue cells readily.[1] Sulfapyridine, according to Hansen,[2] is found in higher concentrations in plasma than in blood cells to an extent greater than could be explained by differences in water content. We have found that sulfathiazole shows a similar though more marked disproportion between concentrations in cells and plasma when analyzed by the method of Bratton and Marshall.[3] In 13 convalescent patients the mean ratio of whole blood-free sulfathiazole to serum-free sulfathiazole was 0.74 ± 0.09 standard deviation. Ten patients treated with sulfapyridine showed a ratio of 0.89 ± 0.07. Comparison of the means by the method of Student for small samples indicates that this difference between the drugs is highly significant.

When the concentration of sulfathiazole in the blood cells was calculated, the ratio to the concentration in blood plasma was 0.437 ± 0.19 as compared with 0.645 ± 0.18 for sulfapyridine, likewise a significant difference. Individual variations in the distribution of both drugs between cells and plasma were marked. Administration of the drug was continued for 2 days. No difference in distribution between cells and plasma could be detected on the second as compared with the first day.

---

* Supported in part by the Pennsylvania State Department of Health, Pneumonia Control. We are indebted to Dr. George A. Harrop, Squibb Institute for Medical Research, New Brunswick, N. J., for the sulfathiazole used in this study.

[1] Marshall, E. K., Jr., Emerson, K., Jr., and Cutting, W. C., *J. Pharm. and Exp. Therap.*, 1937, **61**, 196.

[2] Hansen, L., *J. Lab. Clin. Med.*, 1940, **25**, 669.

[3] Bratton, A. C., and Marshall, E. K., Jr., *J. Biol. Chem.*, 1939, **128**, 537.

A similar comparison of the acetylated fractions of both drugs of the same patient showed an average ratio of 0.755 ± 0.30 of acetylated sulfathiazole in whole blood to that in serum while that of sulfapyridine was 0.973 ± 0.48. However, this difference is not statistically significant. Low concentrations of acetylated drug made the error of analysis appreciable and the failure to demonstrate a difference may be explained by this fact.

Sulfathiazole treated patients have shown considerably lower concentrations of drug in whole blood than patients treated with equal amounts of sulfapyridine. This difference between the drugs is explained in part by the smaller amount of sulfathiazole present in blood cells. The use of plasma or serum for relating drug concentration to therapeutic activity thus is to be preferred.

Our observations are in accord with those of Sadusk, Blake, and Seymour[4] who found in cerebrospinal fluid less sulfathiazole than sulfapyridine when equivalent amounts of drug were administered. It appears, therefore, that other types of cells besides red blood cells are likewise less permeable to sulfathiazole than to sulfapyridine.

Summary. Sulfathiazole is present in human red blood cells in lower concentrations than is sulfapyridine employed under identical conditions.

11668

Carbon Dioxide Tension and its Relation to the Quiescence of Spermatozoa *in vivo*.

L. B. SHETTLES.* (Introduced by N. J. Eastman.)

From the Department of Obstetrics, the Johns Hopkins University and Hospital.

It is generally agreed (Hartman,[1]) that spermatozoa are immotile within the male generative tract. Hartman found the tubules of the cauda epididymidis of the bat filled with spermatozoa during the winter, but he could observe no movement by means of transmitted light. It is his opinion that when spermatozoa are massed, *e.g.,* in the cauda epididymidis, carbon dioxide production maintains the environment on the acid side, thus immobilizing them.

---

[4] Sadusk, J. F., Jr., Blake, F. G., and Seymour, A., *Yale J. Biol. Med.*, 1940, **12**, 681.

* Fellow, National Committee on Maternal Health.

[1] Hartman, C. G., Ovulation, fertilization and the transport and viability of eggs and spermatozoa, Chapter IX in Allen's *Sex and Internal Secretions*, 1939, p. 630.

Roemmele,[2] Belnoschkin,[3] and Granzow,[4] however believe that spermatozoan motility commences in the testis and progressively increases in the excretory tubules. Redenz[5] reports that the secretion of the rete tubules is rich in electrolytes which are favorable for motility, but that the epididymis contains a colloidal suspension which is poor in electrolytes and unfavorable for motility.

In 1940, the present writer[6] found that, *in vitro,* carbon dioxide produces complete immotility of human spermatozoa within a few minutes, the exact time varying with the donor, the freshness of the specimen, the frequency of ejaculation, and certain other factors as yet unknown. The effect of this gas was due neither to its acid character nor to the anoxia produced by it. Motility of the cells was restored when the carbon dioxide was replaced by nitrogen, air, or oxygen immediately after all movement had ceased. However, fewer and fewer cells were capable of restoration of motility after each additional exposure to carbon dioxide.

The present study deals with the question: are high carbon dioxide tissue tensions the actual cause of the quiescence which spermatozoa exhibit *in vivo?* We determined: (1) The carbon dioxide content of whole fresh testis, epididymis, vas deferens, liver, and skeletal muscle of the rat. (2) The concentration of carbon dioxide required to immobilize spermatozoa *in vitro*. (3) The toxicity of carbon dioxide in varying concentrations. (4) The length of time which spermatozoa can be immobilized by carbon dioxide and yet be resuscitated later.

The method of Danielson and Hastings[7] was used for the determination of tissue carbon dioxide. The tissue studied was placed in a side tube connected to a Van Slyke apparatus within 30 seconds after the dissection was begun. The carbon dioxide was liberated by the addition of acid and the application of heat at reduced pressure. The carbon dioxide produced was absorbed by alkali in the reaction chamber. The technic used with blood by Van Slyke and

---

[2] Roemmele, O., *Zool. Jahrb. Abt. allg. Zool. u. Physiol.,* 1927, **44**, 85.

[3] Belnoschkin, B., *Z. f. Biol.,* 1932, **92**, 542.

[4] Granzow, J., *Arch. f. Gynäk.,* 1932, **198**, 149.

[5] Redenz, *Bioch. Ztschr.,* **257**, 234.

[6] Shettles, L. B., *Am. J. Physiol.,* 1940, **128**, 408.

[7] Danielson, I. S., and Hastings, A. B., *J. Biol. Chem.,* 1939, **130**, 349.

TABLE I.

CO_2 Content of Various Tissues of the Rat in mM per Kilo.

Animal No.	Liver	Muscle	Testis	Epididymis	Vas deferens
1	16.0	11.2	6.7	5.7	7.5
2	15.8	10.8	3.5	6.3	4.7
3	18.7	9.2	6.4	4.9	4.5
4	18.0	12.9	5.0	5.8	6.4
5	13.3	10.5	3.9	6.0	8.0
6	17.0	11.4	6.6	4.8	9.3
7	15.9	13.9	4.1	5.9	8.7
8	17.5	10.7	2.9	8.3	6.6
9	16.8	14.0	7.3	7.3	5.6
10	14.3	13.9	5.8	8.0	9.8
Mean	16.5	11.9	5.2	6.3	7.1
Standard error	±2.7	±2.7	±2.2	±1.3	±3.1

Neill[8] was then employed to determine the carbon dioxide contained in the alkali. A ferric fluoride solution (1 volume of 4% NaF + 1 volume of 10% $Fe_2(SO_4)_3$) was used to prevent further production of carbon dioxide by enzymatic action. The results obtained are given in Table 1.

The average carbon dioxide content of the liver, muscle, testis, epididymis, and vas deferens was 16.5, 11.9, 5.2, 6.3, and 7.1 mM per kg, respectively. The standard error ranged from 1.3 to 3.1. The content of the gas in the liver was over 3 times as great as in the testis and over twice as great as in either epididymis or vas deferens. Also, the carbon dioxide tension of skeletal muscle was over twice as great as that of the testis and considerably greater than that of the epididymis or vas deferens. The relatively low carbon dioxide content of testis, epididymis, and vas deferens will become of more interest when it is compared with the *in vitro* concentration of the gas required for immobilization. It will also be noted that of the generative organs the carbon dioxide content of the vas deferens was greatest, the epididymis less, and the testis least.

The *in vitro* effect of carbon dioxide on spermatozoa was studied in the following manner: Fluid from the vas deferens and physiological saline solution were mixed in equal volume. A hanging drop of the mixture with constant volume was placed on a cover glass. This glass was sealed with vaseline on a small gas chamber. The chamber was provided with an inlet and outlet so that a known mixture of carbon dioxide and oxygen could be passed through. This mixture was obtained by means of flow meters. Various concentrations of carbon dioxide were tried until a threshold for immobilization of the spermatozoa was obtained. The spermatozoa were kept quiescent for various lengths of time in different concentrations of the gas. Then pure oxygen was passed through the compartment of the chamber and any resuscitation noted. Control tests were made in the same way without carbon dioxide. These experiments were conducted at room temperature. The results are presented in Tables II and III.

---

[8] Van Slyke, D. D., and Neill, J. M., *J. Biol. Chem.*, 1924, **61**, 523.

Table II shows that the concentration of carbon dioxide necessary for the immobilization of spermatozoa in a carbon dioxide-oxygen

TABLE II.
In Vitro Concentration of CO_2 Required for Immobilization of Spermatozoa.
Immobilization, +; no immobilization, —

	Concentration of CO_2 in carbon dioxide-oxygen mixture in %			
Animal No.	15	18	20	25
1	—	—	+	+
2	—	+	—	+
3	—	—	+	+
4	—	—	+	+
5	—	+	—	+
6	—	—	+	+
7	—	+	+	+
8	—	—	—	+
9	—	—	+	+
10	—	+	+	+
% immobilized	0	40	70	100

TABLE III.
Relation Between Concentration of Carbon Dioxide, Time of Immobilization and Revival of Spermatozoa with Pure Oxygen.
Resuscitation, +; no resuscitation, —.

	Concentration of CO_2 in carbon dioxide-oxygen mixture in %											
	20				60				100			
	Time of immobilization in minutes											
Animal No.	3	5	10	15	3	5	10	15	3	5	10	15
1	+	+	+	+	+	+	+	—	+	—	—	—
2	+	+	+	—	+	+	+	—	+	+	—	—
3	+	+	+	—	+	+	+	—	+	+	—	—
4	+	+	+	+	+	+	+	—	+	—	—	—
5	+	+	+	—	+	+	+	—	+	+	—	—
6	+	—	—	—	—	—	—	—	—	—	—	—
7	+	+	+	+	+	+	—	—	—	—	—	—
8	+	+	+	+	+	—	—	—	+	+	—	—
9	+	+	+	+	+	+	+	—	+	—	—	—
10	+	+	—	—	+	—	—	—	+	—	—	—
% revived	100	90	80	50	90	70	60	0	80	40	0	0

mixture is approximately 20%. Campbell[9] reports that the subcutaneous carbon dioxide tension for the rat and guinea pig is approximately 7%. When the carbon dioxide content of the various portions of the generative tract are compared with this *in vitro* concentration of 20%, the former concentrations appear too low to produce quiescence.

Table III demonstrates that the per cent of spermatozoa revived depends upon the length of time that they were immobilized, and also the concentration of the carbon dioxide. Moreover, all concentrations of the gas producing immotility, even the minimum, proved toxic provided the spermatozoa were exposed long enough. If spermatozoa are non-motile *in vivo,* factors other than carbon dioxide must be responsible.

The fact that motility is usually absent after 24 hours *in vitro* exposure at room temperature, indicates that some unusual factors allowing for survival must be present in the intact generative tract. A sample of seminal fluid collected after a week has passed without emission, must contain spermatozoa which are already several days old. And yet there are no significant differences in the length of *in vitro* spermatozoan survival in specimens collected at daily or weekly intervals. Whether the condition within the generative tract which preserves spermatozoa is associated with their immotility is still unknown.

Summary. There is conflicting opinion concerning the motility of spermatozoa within the generative tract. It has been suggested that a high local tension of carbon dioxide prevents them from becoming motile in the epididymis and vas deferens. The carbon dioxide concentration of various tissues of the rat was studied and found even lower in the generative organs than in liver or skeletal muscle. Furthermore, the concentration of carbon dioxide found in the tissues of the generative tract was much lower than that which is required to immobilize spermatozoa *in vitro.*

---

[9] Campbell, J. A., *Physiol. Rev.*, 1931, **11**, 1.

11669 P

Refined Hyperimmune Rickettsial Sera.

Timothy J. Kurotchkin, J. van der Scheer and
Ralph W. G. Wyckoff.

From Lederle Laboratories, Pearl River, N. Y.

Topping[1] has demonstrated that monkeys and guinea pigs fatally infected with Rocky Mountain spotted fever can be caused to recover by the administration of hyperimmune rabbit serum. This naturally suggests that such serum may have value in the treatment of human cases. The sera prepared by Topping were made by the injection of infectious tick-tissue as antigen. We have found that a serum of high neutralizing titer can also be obtained by injecting rabbits with yolk-sac material prepared according to the method of Cox.[2] We have determined that this can be refined by the same chemical procedures which are useful in purifying antipneumococcal rabbit serum and removing its toxic and reaction-producing elements. In such concentrates the neutralizing titer per gram of protein is increased about 20-fold as compared with the original serum. A similar antiserum and concentrate of high neutralizing value has also been made against the Breinl strain of European typhus. Sufficient quantities of these two sera are now being prepared and refined so that a thorough clinical study of their possible effectiveness in the treatment of Rocky Mountain spotted fever and epidemic typhus fever in man can be made.

11670

Fate of the Lethal Effect of Sublethal Doses of Lanatoside C, Digoxin and Digoxigenin.

Arthur C. DeGraff and Robert A. Lehman.

From the Department of Therapeutics, New York University, College of Medicine.

The availability today of many of the "genuine" and intermediate glycosides and of the genins of the digitalis group has made possible the study of the relationship between structure and pharmacologic action. The importance of this study lies in the possible therapeutic use of the pure substances, thus avoiding the approximations of bioassay, and also in the insight which may be given to the action of the galenicals. With this in mind a comparison has been made of the fate of the lethal effect of sublethal doses of lanatoside C, digoxin and digoxigenin in the cat. This series was chosen because of the chemical relationship of the 3 drugs. Thus, lanatoside C is the "genuine" glycoside as it occurs in digitalis lanata, digoxin the product of partial hydrolytic cleavage of lanatoside C, and digoxigenin is the genin common to both. The chemical relationships are reviewed by Stoll.[1]

---

[1] Topping, N. H., *U. S. Pub. Health Rep.*, 1940, **55**, 41.

[2] Cox, H. R., *U. S. Pub. Health Rep.*, 1938, **53**, 2241.

The method was essentially that originally described by Hatcher[2] and since used by many other workers (Rothlin,[3, 4] van Esveld,[5] Bauer,[6] Gold,[7] Chen,[8] Hoekstra[9]). It consists in injecting intravenously an accurately measured sublethal dose of the glycoside to be studied and, after varying time intervals, infusing ouabain until the death of the animal. It will be noted that this procedure has been applied by various investigators in the study of "cumulation," and "rate of excretion" of the digitaloids. These phrases are avoided since their definition depends upon the arbitrary assumption of a mechanism of action.

As a preliminary step the 3 glycosides were assayed by the usual Hatcher and Brody[10] technic, using a concentration of 1:100,000 for digoxin and lanatoside C, 1:25,000 for digoxigenin, and 1:200,000 for ouabain, and a rate of injection such that all animals died between 30 and 90 minutes after the start of the infusion. Using 10 to 20 cats for each substance, the geometric mean lethal dose of lanatoside C was found to be 0.261, digoxin 0.335 and digoxigenin 0.538 mg per kg. These results do not confirm Rothlin's[11] surprising finding that digoxigenin is more toxic than digoxin, the glycoside from which it is obtained, but are in agreement with Smith[12] and Chen[8] who found digoxin to be the more potent. It will be noted from the table that calculation of the assays in terms of micro-moles per kg accentuates this difference in activity.

---

[1] Stoll, A., *J. Am. Pharm. A.*, 1938, **27**, 761.

[2] Hatcher, R. A., *Arch. Int. Med.*, 1912, **10**, 268.

[3] Rothlin, E., *Munch. med. Wchschr.*, 1933, **80**, 726.

[4] Rothlin, E., *Annale de l'Hospital de la Santa Creu i Sant Pau*, 1935, **9**, 586.

[5] Esveld, L. W., *Arch. f. exp. Path. u. Pharmakol.*, 1931, **160**, 375.

[6] Bauer, H., *ibid.*, 1934, **176**, 65.

[7] Gold, H., *Arch. Int. Med.*, 1923, **32**, 779.

[8] Chen, K. K., Robbins, E. B., and Worth, H., *J. Am. Pharm. A.*, 1938, **27**, 189.

[9] Hoekstra, R. A., *Arch. f. exp. Path. u. Pharmakol.*, 1932, **163**, 35.

[10] Hatcher, R. A., and Brody, J. G., *Am. J. Pharm.*, 1910, **82**, 360.

[11] Rothlin, E., *Schweiz. med. Wchschr.*, 1938, **68**, 1336.

[12] Smith, S., *J. Chem. Soc.*, 1930, **133**, 508.

Initial doses of 0.20 mg per kg for lanatoside C and digoxin and 0.34 mg per kg for digoxigenin respectively were then injected into the femoral vein. The magnitude of the preliminary injection was made as large as possible so that the effect measured would be significantly greater than the variations about the mean, but not so large as to kill any of the animals within 24 hours. The arbitrary adjustment used by Gold[7] for difference in susceptibility was thus avoided.

The variations in the lethal effect were determined for 3 or 6 and 24 hour intervals. Eight to 10 cats were sacrificed for each time interval. The concentration of the ouabain solution for the final titration was 1:200,000 in saline and the rate of injection was one to 2 cc per minute. No general anesthetic was used at any time. Rothlin,[4] Bauer,[13] Heubner and von Nyary[14] have objected to the Hatcher procedure on the ground that after a large but sublethal dose of a digitaloid, cats lose considerable weight and develop pathological changes in the myocardium. They maintain that when ouabain is infused 2 to 14 days after an initial injection, the amount required is not a function of the time interval, nor of the magnitude of the preliminary dose, but rather of the extent of the pathologic changes in the heart and the state of nutrition of the animal. For time intervals of less than 48 hours, however, no pathology of the heart was observed and the state of nutrition of the animal would not be expected to influence the experiments. It is probable, therefore, that the method of Hatcher is capable of following early changes in the lethality of these drugs in the animal body.

The results may be calculated a number of ways without altering the conclusions. The following expression,

$$\frac{100[(\text{Initial Dose}) + (\text{Final Dose}) - 1]}{(\text{Initial Dose})}$$

gives the percentage of the lethal effect of the initial dose which is dissipated after the stated interval, where the dosages are expressed as fractions of the Hatcher and Brody lethal dose (Table). The animals varied in weight from 2.5 to 4.0 kg. The mean weight of the entire 112 cats was 3.1 kg. The results are shown graphically in the figure.

Conclusions. The lethal effect of lanatoside C dissipates rapidly

---

[13] Bauer, H., and Fromherz, K., *Klin. Wchschr.*, 1933, **12**, 973.

[14] Heubner, W., and v. Nyary, A., *Arch. f. exp. Path. u. Pharmakol.*, 1934, **177**, 60.

TABLE I.
Geometric Mean Lethal Dose of Lanatoside C, Digoxin and Digoxigenin by the Hatcher and Brody Assay.

			Lethal dose		
Glycoside	Empiric formula*	Molecular wt	Mg per kg	Micro mols per kg	Values in the literature mg per kg†
Lanatoside C	$C_{49}H_{76}O_{20}$	984	0.261	0.265	0.255 Rothlin[11] 0.280 Rothlin[11]
Digoxin	$C_{41}H_{64}O_{14}$	780	0.335	0.430	0.285 Rothlin[4] 0.22 Chen[8] 0.442 White[15]
Digoxigenin	$C_{23}H_{34}O_5$	390	0.538	1.38	0.253 Rothlin[11] 0.473 Chen[8]
Ouabain	$C_{29}H_{44}O_{12}$	584	0.102	0.171	0.10 to 0.12 numerous observers

*Stoll.[1]

†All of these values are for the cat by intravenous infusion but some were obtained by modifications of the Hatcher and Brody technic.

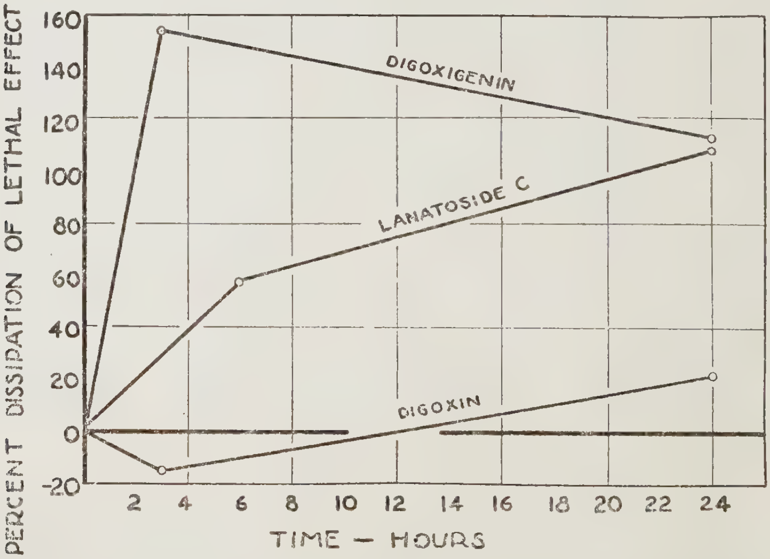


FIG. 1.
Per cent dissipation of the lethal effect of sublethal doses of lanatoside C, digoxin and digoxigenin with time.

during the first 24 hours after intravenous injection in the cat. The lethality of digoxin, however, actually increases during the first three hours and is still quite strong after 24 hours. This very considerable qualitative difference between the action of the "genuine" glycoside and the product of its partial hydrolytic degradation appears not to have been emphasized before. As would be expected, the effect of digoxigenin completely disappears in 24 hours. At 3 hours, however, digoxigenin causes an increased tolerance for ouabain. Further investigation is needed to determine the significance of this finding.

---

[15] White, A. C., *J. Pharmacol.*, 1934, **52**, 1.

11671

Cultivation of Virus of Equine Encephalomyelitis in Serum-Ultrafiltrate and Buffered Salt Solution (Simms).*

MURRAY SANDERS AND ELEANORA MOLLOY.†

From the Departments of Bacteriology and Dermatology, College of Physicians and Surgeons, Columbia University, New York City.

Propagation of a virus in protein-free serum-ultrafiltrate cultures was first accomplished with the agent of lymphogranuloma venereum.[1] The same technic has since been successfully applied to St. Louis encephalitis[2] and a murine strain of poliomyelitis.[3] In the previous studies emphasis was placed upon the stability of the serum-ultrafiltrate cultures as manifested by consistent pH's ranging approximately between the neutral point and 7.6, by persistent viability of tissue cells for long periods of time and proportionate maintenance of high viral potencies. In 2 of the 3 viruses mentioned (*Lymphogranuloma venereum* and St. Louis encephalitis) it was noted that better growth was obtained at room temperature (approximately 20-30° C.) than in the incubator at 37° C. In the case of the third virus, that of murine poliomyelitis, the unusual level of potency of 1 to one billion was obtained with the clear supernatant fluid of cultures kept at 37° C.

The present communication deals with the application of the serum-ultrafiltrate technic to the cultivation of the virus of equine encephalomyelitis (Western and Eastern strains).

The virus of equine encephalomyelitis apparently can be grown

---

* This work was aided by a grant from the John and Mary R. Markle Foundation.

† Fellow in Bacteriology.

[1] Sanders, M., *J. Exp. Med.*, 1940, **71**, 113.

[2] Molloy, E., PROC. SOC. EXP. BIOL. AND MED., 1940, **44**, 563.

[3] Jungeblut, C. W., and Sanders, M., PROC. SOC. EXP. BIOL. AND MED., 1940, **44**, 375.

at will in Tyrode fluid preparations[4, 5] and on the chorioallantoic membrane of the developing hen's egg.[6] Potencies of 10^{-5} for the flask cultures and 10^{-6} for the chorioallantoic preparations have been reported. In more recent work[7] titers as high as 10^{-8} and 10^{-9} have been obtained from whole developing egg preparations (minced embryo plus chorioallantois). The highest titers occurred when the Eastern strain was used.

Materials and Methods. Tissue cultures were prepared in rubber stoppered 50 cc Erlenmeyer flasks by adding minced embryonic chick tissue (with eyes, beak and limbs removed) to 10 cc of serum-ultrafiltrate diluted 1 in 3 with Simms' salt solution.[1] In order that the effect of the serum ultrafiltrate on the virus might be evaluated, similar preparations were made which contained only the buffered salt solution. Both types of cultures were inoculated with 0.1 cc of a 1:10 mouse brain virus suspension (Western strain) and parallel series of each type maintained at room temperature and at 37° C. Passages were made by transferring 0.1 cc of the clear supernatant fluid every 3 days. After intervals of 3, 6, 10, 15, 20, or 30 days incubation, potency tests of various culture generations were carried out by intracerebral inoculation of groups of 4 mice (12 to 15 g) with 0.03 cc of serial tenfold dilutions of the supernatant fluid of the culture (Tables I, II, III, IV). The endpoint in these titrations was taken as the last dilution causing characteristic symptoms and death in 50% of the inoculated mice. The identity of the virus was assured by two neutralization tests with a known antiserum, carried out with the 5th and 28th culture passages.

TABLE I.
Equine Encephalomyelitis (Western Strain) in Serum-Ultrafiltrate Chick Tissue Cultures Maintained at Room Temperature (20-30°C).

Culture generation	Final dilution activity of culture fluid after varying periods					
	3 days	6 days	10 days	15 days	20 days	30 days
7	10^{-5}					
13	10^{-6}					
20	10^{-6}	10^{-5}	10^{-4}	10^{-5}		at least 10^{-2}
24	10^{-5}					10^{-2}
30	10^{-6}	10^{-6}	10^{-5}		10^{-3}	10^{-1}
33	10^{-7}	10^{-5}	10^{-4}	10^{-3}		10^{-2}
35	10^{-6}	10^{-6}	10^{-5}	10^{-4}		

Blank space—no titration done.

---

[4] Cox, H. R., Syverton, J. T., and Olitsky, P. K., PROC. SOC. EXP. BIOL. AND MED., 1933, **30**, 896.

[5] Olitsky, P. K., Cox, H. R., Syverton, J. T., *J. Exp. Med.*, 1934, **59**, 159.

[6] Higbie, E., and Howitt, B., *J. Bact.*, 1935, **29**, 399.

[7] Beard, J. W., Beard, D., and Finkelstein, H., *J. Immunol.*, 1940, **38**, 117.

TABLE II.

Equine Encephalomyelitis (Western Strain) in Serum-Ultrafiltrate Chick Tissue Cultures Maintained at 37°C.

Culture Generation	Final dilution activity of culture fluid after varying periods			
	3 days	6 days	10 days	15 days
13	10^{-5}			
20	10^{-5}	10^{-4}	10^{-1}	0
24	10^{-4}			0
30	10^{-5}	10^{-3}	10^{-2}	10^{-1}
33	10^{-5}	10^{-3}	10^{-1}	0

TABLE III.

Equine Encephalomyelitis (Western Strain) in Salt-Solution (Simms) Chick Tissue Cultures Maintained at Room Temperature (20-30°C).

Culture Generation	Final dilution activity of culture fluid after varying periods				
	3 days	6 days	10 days	15 days	20 days
5		10^{-5}	*	*	*
6	10^{-5}	10^{-5}	10^{-3}	10^{-2}	*
9	10^{-5}	10^{-4}	10^{-3}		10^{-1}
16	10^{-6}	10^{-5}	10^{-2}	*	*
18	10^{-6}	10^{-5}	*	*	*
21		10^{-4}	*	*	*

*Discarded because of excess acid formation (pH about 5).

TABLE IV.

Equine Encephalomyelitis (Western Strain) in Salt-Solution (Simms) Chick Tissue Cultures Maintained at 37°C.

Culture Generation	Final dilution activity of culture fluid after varying periods			
	3 days	6 days	10 days	15 days
6	10^{-4}	10^{-4}	*	*
9	10^{-5}	10^{-3}	*	*
18	10^{-4}	10^{-3}	10^{-1}	*

*Discarded because of excess acid formation (pH about 5).

Throughout this study, one fact was apparent: the cultures which contained buffered salt solution alone were much less stable than those with the serum-ultrafiltrate mixture. Acid formed in many of the salt solution cultures in a short time and they had to be discarded even before inoculation of virus.[‡] This was particularly true of those cultures kept in the incubator. The serum ultrafiltrate cultures, on the other hand, could be kept at room temperature for at least 10 days before being inoculated with virus. Even if the amount of tissue was somewhat increased above the normal (approximately a ratio of tissue to fluid of 1:100) the pH of these cultures remained constantly between 7.0 and 7.5.

[‡] As has been previously noted,[1] the pH of these cultures can be roughly estimated at all times since the fluid mediums contain 0.1% phenol red, an amount of dye which has been found to be nontoxic for the cells.

The highest potencies, 10^{-5} to 10^{-7} dilution activity, were obtained from the cultures kept at room temperature (Tables I, II, III, and IV). Although the serum-ultrafiltrate cultures at room temperature were only slightly more potent than similar preparations with salt solution, it is clear (Tables I and III) that more frequent titrations were possible with the former group. The greatest difference between the two types of cultures (at room temperature) seems to lie in the ability of the serum-ultrafiltrate preparations to maintain higher potencies over an extended period of time (Tables I and III). The final culture generation tested was the 35th (serum-ultrafiltrate at room temperature). This preparation attained a dilution activity of 10^{-6} after the 3rd day. The same virus content was demonstrated at the end of 6 days, and by the 10th day only a slight decrease in potency was apparent (10^{-5}). Even after 15 days, the culture fluid produced symptoms and death in 50% of the mice injected with a dilution of 1:10,000. It is interesting to note that the same type of preparation kept at 37° C (33rd subculture) reached a potency of 10^{-5} at its highest point. There was a comparatively more rapid loss of potency so that by the 10th day only a trace of virus could be demonstrated and by the 15th day no virus was present.

A preliminary experiment done with the Eastern strain of equine encephalomyelitis in serum-ultrafiltrate yielded a dilution activity of the culture fluid (4th culture generation) of 10^{-7}. The same titer was obtained after 3 days regardless of whether the culture was kept at room temperature or 37° C.

Another type of preparation which was tested with the Western strain consisted of chorioallantoic membrane of 12 to 14 day eggs in serum-ultrafiltrate.[2] The supernatant fluid of such preparations kept at room temperature regularly yielded a dilution activity of 10^{-6} (highest dilution tested).

Preparation of a vaccine. That the fluid of the preparations studied is capable of producing an immunity when made into a vaccine is evidenced by the following experiment. The clear supernatant fluid of a serum-ultrafiltrate culture (titer 10^{-6}) was treated with 0.4% formalin. Guinea pigs receiving 2 weekly intramuscular injections of 1 cc of the final neutralized product were found to be immune to 1,000 to 10,000 m.l.d. of virus. After storage for 4 months in the refrigerator, the same vaccine yielded identical results.

An effort was made to demonstrate the protein content of the vaccine by adding equal parts of saturated ammonium sulfate. No precipitate formed immediately, but after standing in the refrigerator overnight, a faint precipitate could be seen at the bottom of the test tube, suggesting a very low protein content. Further studies on the immunizing capacity and quantitative protein determinations of this type of vaccine are being carried out.

Discussion. Although the potencies which have been obtained in this study are not unusually high for the equine encephalomyelitis virus, it must be remembered that in the developing chick preparation the peak of growth is reached within 9 to 13 hours for both the Eastern and Western strains.[6] In the presence of embryonal death, which occurs regularly by the end of 24 hours, there is a rapid decline of virus potency. Thus, unless the virus is quickly harvested, a poor yield is obtained. However, in the case of the serum-ultrafiltrate cultures maintained at room temperature, the danger of loss of virus due to rapid deterioration is negligible, since there is relatively little decrease in virulence even after 10 to 15 days.

Because the developing egg can be so well impregnated with the equine encephalomyelitis virus, an effective formalized vaccine has been in use for several years. However, the high degree of infectivity is somewhat nullified by the fact that the final vaccine contains as much as 40% chick embryo-tissue-extract.[7] It is therefore not surprising that occasional reactions of varying degrees are seen following the use of chick embryo-tissue-extract vaccines.[8, 9] With the growing need for human vaccination such a problem may well assume greater proportions than it has in the past.

Preliminary tests have shown that the serum-ultrafiltrate from virus infected cultures may be used for the production of a vaccine. Such a product involves a protein-free substrate, minimal manipulation in its preparation, and a good degree of effectiveness and stability. In this respect, it should also be noted that experiments with larger cultures (50 cc) have yielded amounts of virus similar to those obtained from the smaller (10 cc) cultures. There is every reason to believe that large quantities of a serum-ultrafiltrate vaccine can be produced as readily as small quantities.

Conclusions. The Western strain of equine encephalomyelitis has been successfully grown in whole minced chick embryo or chorioallantois in serum-ultrafiltrate and buffered salt solution (Simms') at room temperature and at 37° C.

The highest titers (10^{-5} to 10^{-7}) have been obtained with the clear supernatant fluid of chick and serum-ultrafiltrate cultures maintained at room temperature. The titers of these cultures can be sus-

---

[8] Graham, R., *J. Am. Vet. Med. Assn.*, 1940, **97**, 38.

[9] Schoening, H. W., *J. Am. Vet. Med. Assn.*, 1940, **97**, 39.

tained with little decrease for as long as 10 and 15 days.

The Eastern strain of equine encephalomyelitis has been grown with a dilution activity of 10^{-7} in whole minced chick embryo in serum-ultrafiltrate at room temperature and 37° C.

A vaccine, containing very small amounts of protein, prepared from the clear supernatant fluid of serum-ultrafiltrate cultures protected guinea pigs against 1,000 to 10,000 m.l.d. doses of virus (Western strain).

11672

Characteristics of a Fixed Rabies Virus Cultivated on Developing Chick Embryos.

H. Bernkopf and I. J. Kligler.

From the Department of Hygiene and Bacteriology, Hebrew University, Jerusalem.

In a preceding paper[1] we reported the cultivation of rabies virus in developing chick embryos in 2 series through 9 and 6 successive passages respectively. Since then, one series has been carried through 16 subcultures; the second series has been passaged through 47 subcultures during a period of 18 months and is still being maintained. The purpose of this note is to summarize our observations on the behaviour of this virus to different hosts after prolonged cultivation in the developing chick embryo.

Technic. A fixed virus, presumably derived from the original Pasteur strain, was used for initiating the chick embryo cultures. The first inoculation was made by dropping 0.1 cc of a 10% saline emulsion of infected mouse brain on the allantois immediately over the embryo using the Burnet[2] technique. The same technique was used for subcultures: pieces of the inoculated allantois, or preferably the brains of the embryos of 3 to 6 infected eggs, were ground in a sterile glass mortar and enough saline added to make a 10% emulsion. Each passage material was titrated intracerebrally in mice in tenfold dilutions. In the first 12 passages, 5-day-old embryos were infected; subsequently 6-day-old embryos were used because they were more resistant to the manipulations involved. Passages were usually made 6-11, sometimes up to 14, days after the inoculation.

The infection in the chick embryo. The embryos of earlier as well as later passages developed normally and usually remained alive up to hatching time; in later passages the embryos were sometimes smaller than normal but showed no other apparent defect in development. Virus was already found in embryo brain 4 days after infection and usually reached a maximum after 9 days and usually continued at this concentration until time of hatching; however, virus was not detectable in the brain of inoculated embryos which hatched. The adaptation of the virus to the embryo medium is indicated by the fact that, whereas, original mouse passage virus could not infect embryos 8 days old or over with regularity, and passages were usually negative, virus of the 40th egg passage was transmitted in 3 successive subcultures through 10-day-old embryos.

---

[1] Kligler, I. J., and Bernkopf, H., *Nature*, 1939, **143**, 899.

[2] Burnet, F. M., Sp. Rep. Ser. Med. Res. Coun. Lond., No. 220, 1936.

Embryo brain always gave a higher titer of virus than other tissues; it was usually positive in dilutions of 1:1,000 to 1:10,000. Apparently virus multiplication in the chick embryo is limited; the multiplication of the virus in the chick embryo proceeds 15 days as against 7 days in the mouse, yet the titer in mouse brain is 10 to 100 higher than that in chick embryo brain. The allantois titer was usually lower (1:100 to 1:1,000), but occasionally reached the same titer as the corresponding brain.

The presence of virus in the allantois points to multiplication of this strictly neurotropic virus in a membrane which is supposed to be free of nerve tissue,[2] and raises the question whether fixed rabies virus is able to multiply in embryo tissues other than nerve tissue. Two attempts to cultivate the virus in flask cultures[3, 4] with allantois instead of mouse embryo brain failed to give evidence of multiplication. The virus titre of liver and heart of infected embryos was consistently low (1:10, in one experiment 1:100).

The low pathogenicity of the virus for the developing chick coincided with the scanty histopathological changes found in the infected embryo brain. Degenerative changes of the nerve cells are sometimes observed in brains fixed with Bouin and stained with Hämatoxylin-Eosin or Giemsa; the meninges are frequently richer in cells than normal and there may be slight perivascular infiltration around the smaller blood vessels, the endothelial cells of which appear swollen. In many sections it is difficult to see any changes at all. Negri bodies were never found. No specific changes were noted in the allantois.

Infectivity for mice. Prolonged passage of the virus through chick embryos did not appreciably modify its virulence for mice when injected intracerebrally. Virus of the last chick embryo passages caused the same incubation period of 6-7 days and the same course

---

[3] Webster, L. T., and Clow, A. D., *J. Exp. Med.*, 1937, **66**, 125.

[4] Bernkopf, H., and Kligler, I. J., *Brit. J. Exp. Path.*, 1937, **18**, 481.

of infection as did the original mouse virus. Mice injected intraperitoneally with 1 ccm of a 10% embryo brain emulsion of different passages died in varying percentages according to their age: of 69 mice, 6 weeks old, 7 died, while of 26 mice, 4 weeks old, 5 died of the infection. The surviving mice showed varying degrees of immunity to a subsequent intracerebral infection and complete immunity to an intraperitoneal infection with the original mouse passage virus. This fact was used to demonstrate the serological identity of the chick embryo passage virus with the original fixed virus. Ten 4-weeks-old mice were injected intraperitoneally 0.25 ccm of 10% chick embryo brain emulsion of the 45th passage, 11 animals of the same age served as controls. Ten days later both groups of mice received intraperitoneally 0.25 ccm of a 1:50 brain emulsion of a mouse infected with rabbit brain fixed virus; all the treated animals survived, whereas 7 of the 11 controls died of the infection.

Infectivity for rabbits. In contrast with the unchanged virulence of chick embryo passage virus for mice, that for rabbits was greatly reduced. Brain emulsions of chick embryos of different serial passages were injected intracerebrally into rabbits and simultaneously titrated in mice. Sixth passage virus still produced the disease after the usual incubation period of 6 days, death following on the 8th day. The rabbit injected with brain of the 37th passage died after 13 days. Virus of the 40th passage produced the first signs of trouble after 11 days, paralysis set in after 16 and death after 17 days; mice injected with the brain suspensions of this rabbit were paralysed after 7 days and died 3-4 days later. Virus of the 44th passage with a mouse titre of 1:100 produced no signs of illness in rabbit. Two rabbits injected with 47th passage virus showed the first signs of paralysis after 11 and 13 days respectively; death following on the 14th and 15th day. (Table I). A guinea pig inoculated intracerebrally with the 47th passage embryo brain developed paralysis after 7 days

TABLE I.
Intracerebral Virulence Test of Chick Embryo Passage Virus in Rabbits.

Passage and dilution of chick embryo brain inoculated			Titre i.c. in mice	Onset of paralysis in rabbits after .... days	Death of rabbits after .... days	Remarks
Passage	6	1:10	1:1000	7	8	
’’	37	1:100	1:1000	12	13	
’’	40	1:5	1:1000	16	17	Mice inj. i.c. with rabbit brain paralysed after 7 days
’’	44	1:10	1:100	—	—	Well after 2 mo.
’’	47	1:5	1:500	11	14	
’’	’’	’’	1:500	13	15	

and died 2 days later, the normal course with fixed virus. On the other hand, rabies fixed virus cultivated in Maitland medium with mouse embryo brain shows no decrease in intracerebral virulence for rabbits; the 47th subculture on mouse embryo brain which had the exceptionally low titre of 100 intracerebral mice units, produced complete paralysis in a rabbit 8 days after inoculation. The reduced virulence for rabbits of the chick embryo virus appears, therefore, to be a specific modification.

Summary. After 47 passages through the developing chick embryo, rabies fixed virus shows no enhanced virulence for the chick embryo, as judged by the complete development of the embryo, the relatively low virus titre in the embryo brain and the scanty pathological changes in the brain. The virulence of the chick embryo passage virus for mice and guinea pigs remained unchanged, while that for rabbits appeared reduced considerably, as demonstrated by the prolonged incubation period, and duration of the infection. The specific antigenic character of the chick embryo virus remained unchanged during 47 passages over a period of 18 months.

11673 P

Identity of Prolactin with Water Drive Factor in *Triturus viridescens*.*

C. S. Chadwick. (Introduced by J. C. Burch.)

From the Department of Biology, Vanderbilt University, and the Highlands Biological Laboratory, Highlands, N. C.

Reinke and Chadwick have shown that the factor which induces the water drive in the red eft (land phase) of *Triturus viridescens* originates in the anterior lobe of the hypophysis and, further, that this factor acts independently of the thyroid glands and gonads in effecting the water drive.[1] Indicating the possibility that the water drive factor is similar to or identical with the growth-promoting hormone, Chadwick[2] was able to induce the water drive with injections of a commercial extract of the anterior pituitary which assays a high growth-promoting hormone content.[†] This preparation was not as effective, however, as implants of adult Triturus pituitaries. In view of the fact that the lactogenic hormone has been demonstrated to exist in the pituitaries of certain Amphibia,[3] and that it is available commercially in pure form, it was resolved to attempt the induction of the water drive with this preparation. Positive results would not only shed further light on the identity of the water drive factor but would also indicate a function for the lactogenic hormone in at least one species of Amphibia. To accomplish this end, 45 red efts of *Triturus viridescens,* of varying sex and age, were treated with prolactin and set up for observation according to previously described procedures.

---

* The Prolactin used in these experiments was very generously provided by Dr. H. W. Rhodehamel of the Eli Lilly Research Laboratories.

[1] Reinke, E. E., and Chadwick, C. S., Proc. Soc. Exp. Biol. and Med., 1939, **40**, 691; Reinke, E. E., and Chadwick, C. S., *J. Exp. Zool.*, 1940, **83**, 223.

[2] Chadwick, C. S., Proc. Soc. Exp. Biol. and Med., 1940, **43**, 509.

The crystalline hormone was prepared for injection by shaking a measured quantity in distilled water and then slowly bringing the temperature to 50° C. When completely dissolved the solution had a slightly brownish color. It was cooled to 18° C and injected intraperitoneally. Each eft was given 1/10 cc of solution, containing approximately 2 mg, at each injection and was injected daily for 7 to 10 days. A fresh solution was made up daily.

Of the 45 efts treated, 26 were normal, 13 were thyroidectomized and 6 gonadectomized. The thyroids and gonads were removed from some efts in order to determine whether or not the prolactin could act independently of these glands in inducing the water drive. The efts were brilliant red in color when the injections were started and their size range was from 65 to 90 mm. Six months to 2 years would have been required for efts in this size range normally to seek a water habitat.

Every eft, irrespective of size or sex, voluntarily sought and entered water within 5 to 10 days after receiving the first injection. Controls did not seek water nor would they remain in it when placed there. Every eft except those previously deprived of their thyroids began molting on the third day of injection and continued to molt at 24 to 48 hour intervals until completely adapted to water. The thyroidectomized efts did not molt. Instead, the cornified layer piled up on the body surface giving the efts a very dry, rust-colored skin condition. On entering water, however, this thick cornified layer absorbed water, swelled and lifted off the underlying living epidermis. Large pieces continually floated off into the water until after 3 days in water much the same skin condition prevailed in the thyroidectomized as in the normal and gonadectomized efts. The change towards the adult condition in color, *i. e.,* green dorsally and yellow ventrally, the keeling of the tail and general reactions among all the efts given prolactin was so profound that within 3 to 4 weeks they could be distinguished from adult water-stage controls only by their smaller size.

[†] Antuitrin Growth, Parke, Davis & Co.

[3] Leblond, C. P., and Noble, G. K., PROC. SOC. EXP. BIOL. AND MED., 1937, **36**, 517.

The effect of prolactin on the land phase of *Triturus viridescens* has thus been shown to be identical in every way with that of implants of anterior lobes from adult water stages. So far as is known, the return of Triturus to water is essential for reproduction. If prolactin is a normal product of the Triturus pituitary and really is the water drive factor then the process of migrating to water for the purpose of reproduction may be considered a total reaction of the organism similar to—or analogous with—the maternal behavior and broodiness which prolactin induces in organisms much higher in the phylogenetic scale. This possibility is quite in line with the statement of Riddle and Bates[4] that "lactogenesis is a response to prolactin which excites also—in both sexes—additional responses more ancient phylogenetically and more significant generally".

Summary. Prolactin induces the water drive in normal, thyroidectomized or gonadectomized land phases of *Triturus viridescens* within 10 days, irrespective of their size or sex. It induces repeated molting in efts with normal thyroids but not in efts deprived of their thyroids. It causes also the rapid assumption of the adult coloration and finally the appearance of adult structural characteristics.

11674 P

Emulsions of Ethylaminobenzoate as Topical Anesthetics.

R. BEUTNER AND K. R. BEUTNER.

From the Department of Pharmacology, Hahnemann Medical College, Philadelphia.

Ethylamino benzoate, the mother substance of procaine, butyn, and many other local anesthetics, is in itself a valuable surface anesthetic, but is difficult to use on account of its insolubility in water and its inability to form water soluble salts. The solutions in alcohol are too irritant, the solutions in fats too inactive. Attempts were therefore undertaken to use ethyl amino benzoate (also known as benzocaine) in the form of a finely divided suspension in watery colloid solution. However, the use of various hydrophilic colloids

---

[4] Riddle, O., and Bates, R. W., *Sex and Internal Secretions*, 1939, 1089.

per se proved *inadequate* to keep this drug in finely divided suspension and in a pharmacologically active state. Such a suspension even when containing 5% of benzocaine failed to produce any anesthesia when applied to a rabbit's cornea. A solution of this problem was found by first suspending a resin, preferably a phenol resin or mastix in a watery solution of acacia and subsequently adding "benzocaine" to it. In this manner a stable suspension was formed in which the benzocaine functioned as a potent local anesthetic.

While in the absence of the resin "benzocaine" settles completely in about a day, the resin containing suspension settles only to a slight extent. More important is the lasting local anesthetic effect which it produces particularly if phenol resins are used. Upon instilling a 1% benzocaine suspension containing about 4% resin on a rabbit's cornea a superficial local anesthesia lasting from 1 to 5 hours was obtained.

However, some of these resins had in themselves a feeble effect upon the cornea, either slightly irritant or anesthetic. In order to eliminate this effect, one eye of a rabbit was instilled with a 4% resin solution without benzocaine while the other eye was treated with the same emulsion plus 1% benzocaine, and the time of extension of the local anesthesia was noted. This addition of benzocaine extended the time of superficial local anesthesia of the rabbit's eye at least one hour in one case, but over 2 and up to 5 hours in 4 other cases. In these experiments the dispersing agent used was a phenolic resin which has a slightly anesthetic action *per se*.

In another set of experiments the anesthetic effect of finely powdered benzocaine dusted upon a rabbit's cornea was compared with the local anesthetic effect of benzocaine *1%* suspended by means of resin as described. The massive dusting of the eye with the pure local anesthetic proved to be far less efficient than the application of the dilute emulsion; to quote just 2 of several experiments: the emulsion instilled on the left eyes of 2 rabbits gave an anesthesia lasting 3 hours and 50 (or 53) minutes; the right eyes of the same animals were dusted as described but were anesthetized for only 1 hour and 26 to 27 minutes

Even less active than the dust was a 1% benzocaine solution in corn oil (which is an almost saturated solution). This gave only 46 to 47 minutes of anesthesia in the left eyes of 2 rabbits while the right eyes of the same animals, treated with the 1% benzocaine emulsion, remained anesthetized for over 4 hours. Alcohol solutions of benzocaine were not tried on account of the overwhelming irritation they cause to the eye.

It is important that a 1 or 2% procaine hydrochloride solution was found to be even much less active than the oil solution just described, under the same conditions, giving an anesthesia of less than 10 minutes or none at all in some cases. This means that "benzocaine", when rendered soluble by the attachment of a diethyl amino group plus HC1 (which is procaine HC1) is a less active surface anesthetic than unmodified benzocaine if it is only finely divided, suspended in water.

Conclusions. By a suitably fine dispersion in water benzocaine can be rendered efficacious as a topical anesthetic, to a higher degree than when powdered on the mucous membrane in pure form, or when dissolved in oil, or when rendered water soluble as a diethyl amino compound (=procaine).

11675

Immunological Studies on Intestinal Theiler's Virus, and Its Relation to Poliomyelitic Virus.*

PETER K. OLITSKY.

From the Laboratories of the Rockefeller Institute for Medical Research, New York City.

The properties of Theiler's virus (mouse encephalomyelitis, or so-called "mouse poliomyelitis") as it is found in the intestines of practically all normal albino mice of young adult and mature age, or as it occurs in mice having the spontaneous or experimental disease, have recently been described.[1, 2] The similarity of Theiler's virus to that of human poliomyelitis in many characteristics has been pointed out[1, 2] although Theiler[3] has shown that there is no relationship between them in immunological reactions or in host-susceptibilities. Now that there is at hand the intestinal form of the mouse virus, it was thought desirable to make further attempts to determine the relation of this agent to the virus of poliomyelitis. To this end, studies on the possible existence of active and passive cross immunity were undertaken as well as on the pathogenesis of the mouse virus in *rhesus* monkeys.

---

* The writer thanks Dr. M. Theiler for his cooperation, Dr. E. H. Lennette for a sample of pooled, monkey poliomyelitic antiserum, and Mr. P. Haselbauer for technical assistance.

[1] Olitsky, P. K., PROC. SOC. EXP. BIOL. AND MED., 1939, **41**, 434; 1940, **43**, 296; *J. Exp. Med.*, 1940, **72**, 113.

[2] Theiler, M., and Gard, S., *J. Exp. Med.*, 1940, **72**, 49, 79.

[3] Theiler, M., *J. Exp. Med.*, 1937, **65**, 705.

It is agreed[4] by many investigators that human poliomyelitic virus, passaged for long periods of time in monkeys, is not transmissible to white mice. Our own experiences include observations on more than 275 mice (Rockefeller Institute, albino strain) of ages varying from 15 days to 9 months, and a wide variety of methods of inoculation of the virus. The strains of the latter which were employed, MV and Philadelphia 1932, had been maintained for several years by serial passages in *rhesus* monkeys. The mice were exposed to the virus in the following ways: a) by intranasal instillation (10 to 20% suspension of fresh CNS tissue, given in 3 daily doses; or each dose was preceded by a nasal wash with salt solution, horse serum, phosphate buffer at pH=5, or ether; or the first dose was immediately preceded by intracerebral injection of starch solution or scarification of the nasal mucosa); b) by subcutaneous injection (1 cc simply, or this amount just after starch solution had been given intracerebrally); c) by intraäbdominal inoculation (1 cc once or 3 doses introduced on alternate days); and d) by intracerebral inoculation simply, also followed by 4 brain passages in mice at 24-hour intervals. In addition, the inoculated mouse brain of d) was removed on the 2d day and transferred to monkeys for 2 consecutive monkey-passages. The outcome of all these experiments was negative.

The sera obtained from mice 1 month after the administration of poliomyelitic virus by intranasal, subcutaneous, intraäbdominal, and intracerebral routes were pooled (about 10 sera representing each method of inoculation in a pool) and each respective lot was tested for neutralizing antibodies against MV and Philadelphia virus. 1 part of 5% active filtrate of affected monkey cord and 4 parts of mouse serum were mixed; the serum-virus mixtures were kept for 2 hours at 37° C and overnight in the refrigerator. Then 1 cc was injected intracerebrally in monkeys, of which there were 13 test animals and 8 controls. Virus-neutralization could not be detected by these means. In another experiment, 20 mice 20 to 25 days of age received intraäbdominally 3 times on alternate days 0.5 to 1 cc of 10% MV and Philadelphia strains of poliomyelitic virus; this dose was larger than that which induced antibodies in monkeys.[5] Serum collected 30-40 days after the last dose was pooled and tested for the presence of neutralizing antibodies against intestinal Theiler's virus as it occurs in normal mice; and, as an elaboration of controls of the test, also against this virus as present in the CNS of mice acutely ill with encephalomyelitis induced by injection of intestinal contents of normal mice. The neutralization tests were made as just described and the results, as noted in more than 300 mice inoculated intracerebrally with 0.03 cc of serum-virus mixtures (virus in decimal dilutions), disclosed that a) the sera of normal, untreated mice of the same age as that at which the "immunized" mice were bled, *i. e.*, about 2 months, neutralized between 10 and 100 infective cerebral doses of Theiler's virus deriving from intestinal contents or from CNS tissue. b) The sera of mice which had been repeatedly injected with the poliomyelitic viruses showed no greater degree of neutralization than those of normal, uninoculated animals. c) Normal monkey serum had no capacity to inactivate Theiler's virus.

---

[4] For a recent review of the literature see Toomey, J. A., *J. Pediatrics*, 1940, **16**, 519.

[5] Olitsky, P. K., and Cox, H. R., *J. Exp. Med.*, 1936, **63**, 109.

From the results of the foregoing experiments, it can be concluded that the MV and Philadelphia, monkey-passaged strains of poliomyelitic virus are, as determined by the methods here described, not only non-pathogenic in albino mice but also fail to induce antiviral bodies against the homologous strains. Moreover, the serum of uninoculated, normal 2-months-old mice exhibits a certain degree of neutralizing capacity against Theiler's virus as it exists in the intestinal contents of normal mice or in the CNS of such as have experimental encephalomyelitis. Whatever neutralization is observed to occur in sera of animals that have received injections of the poliomyelitic viruses is about equal in degree to that which can be found in untreated mice; hence the poliomyelitic viruses cannot be said to have the power, by themselves, of producing in mice antibodies against Theiler's virus.

The next series of experiments dealt with attempts at detection of neutralizing antibodies in antipoliomyelitic serum, originating from man or *rhesus* monkey, against intestinal Theiler's virus or this virus as found in the CNS of mice having experimental encephalomyelitis. The sera consisted of one sample of pooled, human serum obtained from patients recovered from paralytic poliomyelitis and 7 others collected from monkeys convalescent from paralytic infection induced by MV and Philadelphia viruses. All sera were found to inactivate strains of poliomyelitic virus.† Each sample and Theiler's virus in decimal dilutions (from 10^{-1} to 10^{-4} for intestinal and to 10^{-5} or 10^{-6} for CNS virus) were mixed in equal parts or 4:1 ratios and kept 1 to 2 hours at room temperature or 37° C, and in addition, in most instances, overnight (16 hours) in the refrigerator. Normal monkey serum was used as control. The results of injections of the serum-virus mixtures intracerebrally in more than 450 mice showed no definite, demonstrable neutralization of Theiler's virus.

---

† The convalescent monkey sera were found by Dr. Theiler to inactivate also the Lansing strain which C. Armstrong (*Public Health Rep.*, 1939, **54**, 1719) has succeeded in transmitting to cotton rats and mice.

The final tests related to attempts at transmission to *rhesus* monkeys of Theiler's virus deriving from intestinal contents of normal mice (active to 10^{-3} dilution) and from the tissue of the CNS of mice that developed encephalomyelitis after receiving such contents (active to 10^{-6} dilution). Eight monkeys were injected, 1 cc intracerebrally and 4 to 25 cc intraäbdominally, with a 20% suspension of contents or of nervous tissue. None of them exhibited signs of infection nor, 40 days after inoculation, resistance against active MV or Philadelphia strains of poliomyelitic virus. In addition, intestinal Theiler's virus did not apparently survive in the CNS of *rhesus* monkeys that were injected intracerebrally with it: Cerebral tissue from the site of inoculation plus fragments of spinal cord were removed 1, 2, 4 and 6 days after injection and reinoculated intracerebrally into mice (in 10 for each sample) with negative results. Finally, passage was attempted using nervous tissue obtained from monkeys that had received active intestinal Theiler's virus in the brain. A total of 3 consecutive monkey brain-to-brain passages were carried out. The tissue (site of cerebral injection, basal ganglia, pons-medulla, and cord) was removed on the 6th day after inoculation in all instances, made up in saline solution to 20% suspension and 1 to 1.25 cc given intracerebrally along with 25 cc intraäbdominally. All passages were negative. Under the described conditions, intestinal Theiler's virus could not be successfully implanted in the brain of *rhesus* monkeys.

Discussion and Summary. Attempts at transmission of Theiler's virus to *rhesus* monkeys by means of intracerebral inoculation and, further, by consecutive passage in monkeys, failed; the virus, moreover, was not demonstrable in the CNS of monkeys 24 hours after inoculation. Thus, Theiler's virus has its own traits in monkeys, which fact conforms with Theiler's original conclusion[3] that the mouse virus is not identical with that of human poliomyelitis. Further proof of this finding can be deduced from results of immunological studies. In the foregoing experiments two standard strains of poliomyelitic virus were used, the MV and Philadelphia, both monkey-passaged for several years; what would have been the outcome had strains freshly isolated from human cases of poliomyelitis been studied is, of course, unknown. It is apparent, however, that by the methods here used, such monkey-passaged strains are not only nonpathogenic in the type of mouse which is susceptible to Theiler's virus and which carries the latter in its intestines for a good part of its life, but also fail to produce serum-neutralizing antibodies in such mice against the homologous poliomyelitic viruses and against Theiler's virus. Moreover, antipoliomyelitic sera of human and monkey origin that are capable of neutralizing homologous and other strains of poliomyelitic virus fail to inactivate Theiler's virus. Conversely, serum from mice harboring Theiler's virus does not neutralize the poliomyelitic viruses. On the other hand, serum from normal mice of certain adult age has antiviral bodies against Theiler's virus. In this latter relation, a similarity exists between the virus of Theiler's disease—a natural malady of mice—and that of poliomyelitis of man in that neutralizing antibodies against the latter virus develop with time in man in the absence of clinically apparent disease. It has not as yet been determined, however, whether in man, as in the mouse, there exists the almost universal and prolonged[1, 2] carrier-state—in the intestines or perhaps elsewhere in the host.

11676 P

Isoimmunization in Pregnancy and the Varieties of Isoagglutinins Observed.*

Philip Levine and Eugene M. Katzin.

From the Division of Laboratories, Newark Beth Israel Hospital, Newark, N. J.

The production of immune isoagglutinins following repeated transfusions, although of common occurrence in some animal species, is very rare in man. The first instance of this sort in man was described by Landsteiner, Levine, and Janes.[1]

Levine and Stetson[2] described a case in which an intra-group agglutinin was responsible for severe post-transfusion symptoms, with anuria after the first transfusion. This occurred in a woman who, after retaining a dead fetus for a period of about 2 months, finally delivered a macerated fetus. In view of the activity of the agglutinin at 37° C and because of its gradual disappearance, it was believed that the antibody developed as a result of immunization, and it was suggested that the products of the retained dead fetus served as the

---

* This investigation has been aided by a grant from the Blood Transfusion Betterment Association of New York City.

[1] Landsteiner, K., Levine, P., and Janes, M. L., Proc. Soc. Exp. Biol. and Med., 1928, **25**, 572.

[2] Levine, P., and Stetson, R. E., *J. Am. Med. Assn.*, 1939, **113**, 126.

antigenic stimulus. This suggestion seemed plausible because the cells of the husband, of the same blood group, (who was the donor) were sensitive to the action of the agglutinin. Presumably, the fetus inherited from the father a dominant agglutinogen which was absent in the tissues of the mother, who could thus be immunized.

Our own recent experience and other cases from the literature[3] indicate that the isoimmunization-hypothesis may hold also in certain cases of pregnancy with complications other than the retention of the dead fetus. In each of the cases cited, atypical agglutinins could be demonstrated in the absence of a history of repeated blood transfusions.

In 5 cases[4] there was a history of varying degrees of toxic symptoms during the pregnancy (in 2 of which macerated fetuses were delivered). In one of these cases, the patient had some fever, and rupture of the membranes occurred a few days before delivery, which was normal but followed by a post-partum endometritis. In 3 patients[5] the agglutinins were observed following abortion, one of them of the septic variety. One patient, whose eighth pregnancy resulted in premature separation of the placenta, had stillbirths in the 4 preceding pregnancies.[6] In 2 of the remaining 3 cases there were histories of repeated miscarriages but the present pregnancies, apparently normal, had to be terminated by Caesarian section.[†] In one case the pregnancy was normal except for morning vomiting during the last six weeks; due to lack of progress in labor the delivery was by Caesarian section.[7]

More intensive study of a larger series of cases is required before any interpretation of these complications can be given in terms of the hypothesis of isoimmunization, but there does appear to be a correlation of the complications with the incidence of atypical agglutinins, and one can speculate as to their relationship.

In the seven cases from which data are available the atypical antibody acted on the cells of the father, who was compatible according to the blood groups, a fact which lends considerable support to the hypothesis that, under certain conditions still to be determined, the mother may be immunized by the fetus or the fetal parts of the placenta. In only 2 of these cases were the incompatibilities detected prior to the transfusion; in the remaining cases the transfusions were attempted with resulting shock and in 3 instances the outcome was fatal. Of the 10 cases transfused, 8 showed distinct clinical symptoms and signs of hemolysis of transfused blood (jaundice and varying degree of anuria).

---

[3] Wiener, A. S., and Peters, H. R., *Annals Int. Med.*, 1940, **13**, 2306.

[4] Four of these cases are from our series, one of which will be published with Dr. S. Polayes; the fifth case was reported by Smith and Haman, *Calif. and West. Med.*, 1934, **41**, 157. This patient had toxic symptoms in each of her four pregnancies.

[5] One of these cases was reported by Parr and Krischner, *J. Am. Med. Assn.*, 1932, **98**, 47; another by Johnson and Conway, *Am. J. Obst. and Gynec.*, 1933, **26**, 255; the third case is one of our own.

[6] Zacho, A., *Hospitalstid.*, 1935, **78**, 225; *Z. f. Rassenphysiol.*, 1936, **8**, 1.

[7] Culbertson, C. G., and Ratcliffe, A. W., *Am. J. Med. Sc.*, 1936, **192**, 471; further data were obtained by personal communication.

[†] These cases will be published with Dr. L. Burnham.

Another interesting feature presented in the author's own cases is the variety of isoagglutinins observed. In 4 of the 7 cases the atypical antibody was characterized by a greater degree of activity at 37° C than at lower temperatures. Consequently, the only previously reported case of Zacho[6] can no longer be considered unique. It therefore seems desirable to designate agglutinins of this sort as "warm-agglutinins". In one of our cases the atypical agglutinin reacted at only 20° C and not at 37°. In the 2 remaining cases the isoagglutinin reacted equally well at 37° and at 20°; in one of these, hemolytic reactions were obtained which ran parallel to the results of agglutination with inactivated serum.

It is of further interest that the specificity of one serum containing warm-agglutinins corresponded to the anti-Rh of Landsteiner and Wiener.[8]cf[3] This observation was made in tests done jointly with Dr. Wiener.

The clinical and immunological aspects of these cases will be more fully described elsewhere. The material is presented because of the several varieties of isoagglutinins observed and because of the general biological significance of the concept that the mother may be immunized by the fetus.[9]

Finally, attention is called to the analogy of the immunization of the mother by the products of conception and the production of isoagglutinins in rats and mice receiving transplants of certain tumors.[10, 11]

---

[8] This blood factor, demonstrated by means of anti-rhesus blood immune sera, was described by Landsteiner and Wiener, PROC. SOC. EXP. BIOL. AND MED., 1940, **43**, 223.

[9] Indirect evidence for the immunization of the mother by the fetus was furnished by Jonsson, who showed that the anti-A and anti-B content of mothers belonging to group O was specifically determined by the blood groups of the infants, provided they were in groups A or B respectively. (*Acta Path. et Microbiol. Scand.*, 1936, **13**, 424.) Some years previously, Hirszfeld speculated at length on the relationship of heterospecific pregnancy and complications in the mother and infant. (*Konstitutionsserologie and Blutgruppenforschung*, 1928, Springer, Berlin.) The relationship of blood differences in mother and child to the etiology of eclampsia was discussed by Dienst (*Zentralb. f. Gynäk.*, 1905, **29**, 353), Ottenberg (*J. Am. Med. Assn.*, 1923, **81**, 295) and others.

11677 P

Atypical Warm Isoagglutinins.

PHILIP LEVINE,* EUGENE M. KATZIN AND LYMAN BURNHAM.
From the Division of Laboratories of the Newark Beth Israel Hospital, Newark, N. J., and the Englewood Hospital, Englewood, N. J.

Zacho[1] reported an instance of premature separation of the placenta with a transfusion-accident, attributable to an atypical isoagglutinin active on the donor's cells. This agglutinin had the unique property of greater activity at 37° C than at lower temperature. Although Zacho's case was the first and only published instance of this sort, it is likely that hitherto antibodies of this character may have been overlooked. In support of this, case reports are presented of 5 patients whose blood recently investigated were found to contain such agglutinins. A brief discussion of the properties and the origin of these agglutinins forms the basis of this communication.

One of these cases was observed in a patient† (B.M.) transfused 6 times because of bleeding from a duodenal ulcer. Apparently, this agglutinin was induced as a result of the antigenic stimulus of repeated transfusions. No such history could be elicited in the remaining 4 instances, nor in the case of Zacho, all of which were observed in women suffering from a variety of complications of pregnancy.

In one patient (G.B.) who gave a history of 2 miscarriages, the present pregnancy was complicated by death of the fetus during labor. A second patient (H.H.) had 3 consecutive miscarriages and in the present pregnancy delivery was by Caesarian section because of uterine inertia. A third patient (D.D.) who suffered from persistent vomiting from the fourth to the seventh months had fever

---

[10] Gorer, P. A., *J. Path. and Bact.*, 1937, **44**, 691.

[11] Lumsden, *Am. J. Cancer*, 1938, **32**, 395.

* Aided by a grant from the Blood Transfusion Betterment Association of New York City.

[1] Zacho, A., *Z. f. Rassen phys.*, 1936, **8**, 1.

† B. M. is a patient of Dr. Abell, Louisville, Kentucky. The study of this blood was made possible by Dr. D. C. Bull.

TABLE I.
Tests with Serum B.M. (Group O).

Read after 1½ hours at °C	Red Blood Cells of Group O 1	2	3	4	5	6
20	0	0	tr.	f.tr.	0	0
25	±	tr.	±	tr.	0	0
37	+	+	+±	+	±	0

TABLE II.
Tests with Serum H.H. (Group A).

Read after 1½ hours at °C	Red Blood Cells of Group					
	O 11	O 12	O 13	A 14	A 15	A 16
20	0	0	±	0	±	0
37	+	0	+±	+	+±	+

and premature rupture of the membranes a few days before delivery which was followed by a parametritis. The fourth patient (A.H.) died of an induced septic abortion. (Zacho's patient gave a history of 4 consecutive stillbirths preceding the last pregnancy.)

The correlation of atypical agglutinins in general with certain complications of pregnancy has been mentioned elsewhere.[2]

The characteristic reactions of these agglutinins in two cases are shown in Tables I and II.

The results with serum G.B. were similar to those indicated in Tables I and II. Serum A.H. gave most contrasting results in tests at 37° C and 25° C at which temperature the serum was practically inactive; at 20° C or lower, stronger reactions of a different specificity were observed indicating the presence also of cold-agglutinins. To a lesser degree, similar observations apply also to serum D.L.

When the test mixtures were centrifuged at low speed for one minute without previous incubation and read by resuspending the sedimented cells, reactions were observed which paralleled those seen in the series incubated at 37° C. Accordingly, the application of this rapid test is sufficiently sensitive for the selection of compatible donors.

In 2 cases tested, the agglutinin was specifically absorbable at 37° C by sensitive but not by resistant blood; absorption by sensitive blood at icebox temperature was incomplete.

There is sufficient evidence that these agglutinins exhibit a specificity different from that of the anti-M, anti-N, or anti-P. The agglutinins in one serum (H.H.) were shown in tests done jointly with Dr. A. S. Wiener to run parallel to the anti-Rh of Landsteiner and Wiener.[3],cf[4]

---

[2] Levine, P., and Katzin, E. M., PROC. SOC. EXP. BIOL. AND MED., 1940, **45**, 343.

These antibodies were designated as warm-agglutinins[2] in order to differentiate them from atypical agglutinins that act at 20° C but not at 37° C, and from those which act equally well at both temperatures.

As to the origin of the warm-isoagglutinins in the pregnant women, it is assumed that they are one of several varieties of antibodies resulting from isoimmunization of the mother by the products of conception.[5] As originally stated by Levine and Stetson, it is believed that the fetus inherits certain dominant agglutinable substances from the father which if lacking in the mother may stimulate her to produce isoantibodies.

11678 P

Action of Sulfapyridine upon Pulmonary Lesion of Experimental Pneumococcal Pneumonia.

W. BARRY WOOD, JR.* (Introduced by J. F. Enders)

From the Department of Bacteriology and Immunology, Harvard University Medical School.

The action of type specific antiserum upon the pulmonary lesion of lobar pneumonia has been described in a previous paper.[1] Pneumonia was produced experimentally in white rats by intrabronchial inoculation of type I pneumococci suspended in mucin. The disease was uniformly fatal in untreated animals, and pneumococci were found to spread through the lung by way of edema fluid at the advancing margin of the lesion. Type specific antiserum penetrated the pneumonic lesion and apparently stopped its spread by agglutinating and immobilizing the invading organisms in the outer edema zone. The fixed pneumococci were then overtaken and were rapidly phagocytized by leucocytes.

Although sulfapyridine has proven beyond any doubt to be effective in the treatment of lobar pneumonia, the mechanism by which recovery is induced is not yet understood. Fully encapsulated living pneumococci are resistant to phagocytosis unless opsonized by specific antibody,[2, 3] and phagocytosis is the only known method by which the host can destroy these organisms.[4] Since chemotherapy often causes a crisis long before antibodies appear in the blood,[5] and sulfapyridine in the usual dosage is mainly bacteriostatic rather than bactericidal,[6] it is not at all clear how the drug brings about the final destruction of pneumococci in the lung.

---

[3] Landsteiner, K., and Wiener, A. S., PROC. SOC. EXP. BIOL. AND MED., 1940, **43**, 223.

[4] Wiener, A. S., and Peters, H. R., *Ann. Int. Med.*, 1940, **13**, 2306.

[5] Levine, P., and Stetson, R. E., *J. Am. Med. Assn.*, 1939, **113**, 126.

* Fellow in the Medical Sciences of the National Research Council.

[1] Wood, W. B., Jr., *Science*, 1940, **92**, 2375, p. 15.

Thirty-eight albino rats were treated with sulfapyridine 6 hours after inoculation. The drug was suspended in 10% gum acacia and introduced into the stomach through a blunt cannula. Two hundred and fifty milligrams of sulfapyridine suspended in 4 cc of gum acacia mixture were given as an initial dose, and half of this amount in 2 cc of acacia was administered thereafter every 12 hours. In uninfected rats no toxic effects were noted after 1 week of treatment.

The protective action of sulfapyridine was tested on a group of 20 rats. Sixteen survived, and when sacrificed at the end of one week, showed sharply demarcated localized lesions obviously arrested by the action of the drug. Eleven of the surviving rats developed bacteriemia 12 to 36 hours after the start of treatment. The bacteriemia in each case was transient and cleared in less than 4 days. The 4 rats that died developed bacteriemia early and succumbed within 3 days, the drug having failed to arrest the spread of the pneumonic process. All untreated animals died.

The manner in which sulfapyridine acted upon the lesion was studied in rats sacrificed in groups of 3 at 6, 18, 42, 66, 96, and 168 hours after the start of treatment. During the first 18 hours the drug had little effect upon the advancing pneumonia. The border of the lesion remained irregular and hemorrhagic, and the many pneumococci in the edema-filled alveoli at the margin indicated that the infection was still spreading rapidly.

At the end of 18 hours there was evidence that the sulfapyridine was beginning to exert its effect. Examination of the pneumococci in the edema zone showed a striking change in their morphology; many were swollen, pleomorphic, and irregularly stained, and a few

---

[2] Ward, H. K., and Enders, J. F., *J. Exp. Med.*, 1933, **57**, 527.

[3] Robertson, O. H., and Van Sant, H., *J. Immunol.*, 1939, **37**, 571.

[4] Heffron, R., *Pneumonia with Special Reference to Pneumococcus Lobar Pneumonia*, The Commonwealth Fund, 1939, p. 151.

[5] Wood, W. B., Jr., and Long, P. H., *Ann. Int. Med.*, 1939, **13**, 612.

[6] Finland, M., Spring, W. C., and Lowell, F. C., *J. Clin. Invest.*, 1940, **19**, 163.

had grown in short chains. Forty-two hours after the start of treatment the edema zone had disappeared completely, the pneumococci at the margin having been overtaken by leucocytes. Most of the organisms were already within phagocytes. The phagocytic reaction was pronounced but resembled that seen deep in the pneumonic lesion in untreated animals[7] rather than that observed in rats treated with antiserum.[8] † Organisms could still be seen within phagocytic cells after 66 hours of treatment but by this time macrophages were numerous in the alveolar exudate and appeared to be taking an active part in destroying the bacteria. On the fourth day no pneumococci could be found in the stained sections, and after one week there was extensive resolution with only macrophages remaining in the rapidly clearing alveoli.

It is significant that phagocytosis occurred in the lungs of animals with bacteriemia and was, therefore, apparently independent of circulating type specific opsonins.[9, 10] The same phenomenon has been observed in untreated animals with bacteriemia.[7] Although phagocytosis in the absence of type specific antibody may be explained by injury to the pneumococcus capsule, the exact mechanism of the process in untreated animals and in those receiving sulfapyridine is not known. Since the natural defense of the lung against pneumococcal infections appears to depend largely upon this type of phagocytic reaction; an attempt is being made to study it further.

Conclusions. Whereas antipneumococcal serum acts immediately and stops the spread of the pneumonic lesion by immobilizing the invading organisms through agglutination, sulfapyridine exerts the same effect more slowly through bacteriostasis. In both cases the final destruction of the pneumococci depends upon phagocytosis, but in animals treated with sulfapyridine the phagocytic reaction may apparently occur in the absence of circulating type specific antibodies.

The author wishes to thank Dr. J. F. Enders for many helpful suggestions and Mr. John Burke for the preparation of microscopic sections.

---

[7] Wood, W. B., Jr., *J. Exp. Med.*, in press.

[8] Wood, W. B., Jr., *J. Exp. Med.*, in press.

† Following serum therapy the reaction was more striking with many more pneumococci in each leucocyte.

[9] Robertson, O. H., Graeser, J. B., Coggeshall, L. T., and Harrison, M., *J. Clin. Invest.*, 1934, **13**, 621.

[10] Terrell, E. E., *J. Exp. Med.*, 1930, **51**, 425.

11679 P

Influence of Sera on Development of the Chick Embryo.

ARNOLD J. GELARIE.

From the Research Laboratory of the Stuyvesant Polyclinic, New York City.

This report deals with conclusions arrived at following studies of embryonal tissues when subjected to the influence of sera, normal and pathological. The author, in collaboration with Barratt,[1] reported the inhibiting effect of embryonal tissues on the growth of implanted cancer in mice. Since then the influence of organ extracts and sera on the development of foetal structures and cell formations has been continuously studied by the writer. During these experiments, it was revealed that cancer serum and serum from pregnant and menopausal women had a marked growth-promoting effect on embryonal tissues.

The fertile hen's egg proved the most satisfactory medium. We began by injecting fertile eggs with sera chosen at random. Later, when abundant sera, from subjects with malignant affections, became available from an authoritative source, systematic examinations were undertaken to discover any selective action of sera on embryonal tissues.

Hundreds of sera were used. In all cases of cancer and sarcoma the clinical diagnosis was confirmed histologically from material obtained by biopsy or surgical procedure.

To determine the optimal conditions, sera, active and inactivated, whether fresh or previously in the refrigerator from 3 to 30 days, in varied dosages, were employed. Injections into the fertile eggs were made at various stages of development of the chick embryo. Before using, the sera were kept in the incubator for 24 hours to test sterility. Control sera were obtained from numerous individuals who exhibited a wide range of pathological conditions.

Technic. The large end of the egg is painted with tincture of iodine, then punctured with a small sterile hand drill. A fine needle is inserted into the center of the egg, and the serum slowly injected. The minute opening is then sealed with a drop of sodium silicate and the egg is replaced in the incubator (38°C) until the end of the hatching period, usually 21 days.

Data ascertained. 1. The optimal time for injection into the fertile egg is the 4th or 5th day of incubation, when, on transillumination, the blood vessels of the developing embryo and the extra-embryonic vascular area can be clearly seen. 2. The quantity of serum required for successful results is from 0.5 to 1.0 cc, preferably 1.0 cc. 3. The age of the sterile serum makes no difference in the results.

---

[1] Barratt, J. O. W., and Gelarie, A. J., *Z. f. Krebsforsch.*, 1913, **13**, 1.

More than 200 cases of cancer and sarcoma, in different stages and grades, were examined. The controls included sera from more than 100 cases, of varied pathological conditions involving practically all organs including acute and chronic diseases. Sera from pregnant and menopausal women were also used for comparative tests. The results of these tests were remarkably consistent.

Conclusions. In 118 out of 122 cases where there were no indications of tumors, injection of the serum was followed by complete arrest of development of the chick embryo, the fertile egg substance undergoing degeneration, usually after the eleventh day of incubation. Confirmation, by waiting until the end of the hatching period, is advisable. The 4 negative results occurred in 2 male cases; one with mitral insufficiency plus migrating phlebitis, and the other with erythema multiforme and trigeminus neuralgia. The other 2 cases were women, one in menopause with atrophic arthritis, and tuberculous ulcers of both corneae; the other with a chronic parenchymatous nephritis.

In marked contrast with these results, when serum from a case of malignant tumor or from a pregnant woman was used, the development of the embryo proceeded normally until a full term chick was hatched. In some cases, the growth was even accelerated, shortening the incubation period, the chicks being noticeably larger and more precocious. Positive results were obtained in 216 cases out of 220. In this group the sera which gave a negative result were 2 with squamous carcinoma of the larynx, one with spindle cell sarcoma of the scapula, and one with melanoma of the face.

The causes of the isolated failures cannot be predicated at the present time. It is to be noted that on the 11th or 12th day, chemical examination of the egg contents for carbohydrates shows marked quantitative differences between eggs injected with cancer serum and those injected with non-cancerous serum. Whatever the explanation, the fact has been established that non-cancerous sera inhibit the development of the chick, while cancerous sera sustain or even accelerate it.

11680

Effect of Acute Anoxia on the Economy of Effort Index in Man,

M. L. FORD, C. L. PETERSILGE, A. F. YOUNG AND C. J. WIGGERS.
From the Department of Physiology, Western Reserve University Medical School.

Sands and DeGraff[1] showed that a degree of anoxia, occasioned when dogs breathe gas mixtures containing less than 12% oxygen, exerts a stimulating effect on ventricular contraction. While thus stimulated, the ventricles eject their blood with a greater economy of effort, as judged by a criterion proposed by Wiggers and Katz.[2] It is important to obtain evidence whether the human ventricle responds in a similar manner.

Wright, Hallaran and Wiggers[3] adapted the procedure of W. and K. to a somewhat rougher estimation of the cardiac economy of effort in man. The procedure merely requires registration of optical subclavian pulses of reliable contour and an estimation of systolic and diastolic brachial pressures by the auscultatory method. Details of the procedure by which the ratio, dynamic effort/static effort, or economy of effort index is derived are given in papers quoted.[2, 3]

Procedure. It was our purpose to determine the changes in such an index while normal subjects were breathing air with progessively diminishing oxygen volumes. For this purpose, medical students rebreathed CO_2-free air from a segment respirometer[4] of 60 liters capacity. The subject was seated and rested for 30 minutes. After connection with the mouthpiece and while breathing room air in this way, a subclavian pulse was recorded by Frank capsules and brachial pressures were determined. Heart rate could be calculated from records. The subject was then connected to the respirometer in which the percentage of oxygen decreased progressively to 14% in about 5 minutes. At this point, the rebreathing mixture was kept constant by slow admission of oxygen while another set of pulse curves and brachial pressures were recorded. In a similar way, the oxygen was reduced to and stabilized at 12 and 10% respectively; recordings of pulses and blood pressure being again taken.

Results. Reliable records and data were obtained in 23 of a larger number of subjects studied. An analysis of these data revealed reactions characteristically found in the majority of normal human subjects during progressive anoxia. The heart accelerated 8 to 32 beats in different individuals, the acceleration being rather more than in those having initial slow rates. The diastolic pressure decreased in all but 2 subjects, in one of which no change and in the other a slow rise was found. The greatest scatter in degree of fall was around 8-15 mm Hg. Systolic pressure remained the same in 5, rose 5-15 mm Hg in 12, fell 3-5 mm Hg in 5, first rose and then fell in 2 subjects. With 2 exceptions a notable increase in pulse pressure occurred.

---

[1] Sands, J., and DeGraff, *Am. J. Physiol.*, 1925, **74**, 416.

[2] Wiggers, C. J., and Katz, L. N., *Am. J. Physiol.*, 1928, **85**, 229.

[3] Wright, G., Hallaran, W. R., and Wiggers, C. J., *Am. J. Physiol.*, 1939, **126**, 89

[4] Burlage, S. R., and Wiggers, C. J., *Am. J. Physiol.*, 1925, **72**, 192.

Tabulations of changes in the *efficiency index* show a general tendency to a slight increase when 14% oxygen is rebreathed and a more definite increase while rebreathing 12% oxygen in all but 3 cases (last 3 in Table I). Of the 20 cases showing a definite increase in the efficiency index, 12 showed a further increase when the oxygen was reduced to 10% (left-hand tabulations, Table I). The other 8 showed some tendency for the index to decrease but it was generally still above the initial values (right hand tabulations, Table I).

Comment. The results indicate that anoxia exerts a stimulating action on the heart in most normal subjects; and aided by lowering of diastolic pressure, the economy with which mechanical energy is utilized is enhanced. The possibility exists that the tendency for the economy of effort to increase or decrease when 10% oxygen is rebreathed may prove valuable as an adjunct to present tests for separating individuals who do and do not tolerate low atmospheric pressures. Unfortunately, opportunity for such correlated studies did not exist.

TABLE I.
Economy of Effort Indices at Various Oxygen Percentages.

Subject No.	Oxygen % in rebreathed air				Subject No.	Oxygen % in rebreathed air			
	20	14	12	10		20	14	12	10
4	.175	.293	.513	.675	12	.240	.288	.497	.472
19	.225	.302	.364	.490	9	.335	.395	.542	.462
18	.240	.483	.480	.512	10	.355	.462	.488	.450
15	.290	.381	.447	.583	16	.370	.488	.593	.512
8	.299	.297	.390	.467	17	.390	.407	.512	.480
7	.332	.401	.575	.670	1	.440	.475	.545	.532
3	.345	.493	.605	.637	5	.518	.512	.590	.510
20	.360	.430	.420	.602	2	.525	.585	.625	.532
6	.362	.450	.450	.515					
14	.390	.437	.518	.683	22	.181	.190	.186	.185
11	.400	.512	.687	.744	23	.285	.273	.280	.240
13	.437	.485	.600	.752	21	.585	.577	.590	.600

Summary. The effect of acute anoxia on the *economy of effort index* of Wiggers and Katz was studied on medical students by allowing them to rebreath decreasing oxygen mixtures and holding these mixtures at 14, 12 and 10% oxygen, respectively, long enough to record subclavian pulses and determine brachial blood pressures leisurely.

In 20 subjects, the index increased during rebreathing of 14 and 12% oxygen; in 12, it increased further when the oxygen percent was reduced to 10; in 8 it began to show slight signs of decreasing. Three subjects showed no significant increase or a slight decrease. The possible use of the method in testing favorable and unfavorable responses of individuals to low barometric pressures is mentioned.

11681

Limitations of Myocardial Recovery from Fibrillation Through Countershock.*

PHILIP DOW AND CARL J. WIGGERS.

From the Department of Physiology, Western Reserve University Medical School.

In revival of the ventricles from fibrillation by use of countershock, stress is generally laid on the limited survival of the central nervous system, and particularly of the cerebrum. It is less commonly stressed that the myocardium likewise can survive the anoxia imposed by complete interruption of the coronary circulation for a very limited time.

Experiments were, therefore, carried out to determine how the duration of fibrillation influences recovery and to estimate the maximum period of fibrillation which may be followed by recovery without the aid of cardiac massage.

Procedure. In dogs under sodium barbital anesthesia, the heart was exposed under mild artificial respiration and aortic pressures were recorded optically by a Gregg manometer. Records were taken before and during fibrillation produced by application of a brief tetanic current and at various intervals after recovery following a series of A.C. countershocks. In these records, systolic and diastolic pressures were measured in representative cycles and plotted. Typical results are shown in Fig. 1.

Results. After fibrillation, less than 10 seconds in duration,

---

* Supported by a grant from the John and Mary R. Markle Foundation.

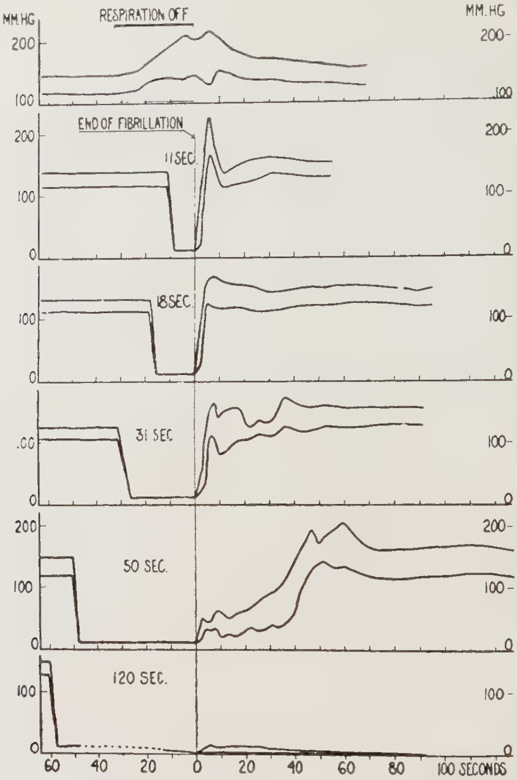


FIG. 1.

Plots showing systolic (upper lines) and diastolic (lower lines) pressures before, during, and after various intervals of ventricular fibrillation.

normal pressures were restored within a few beats. With increasingly longer periods of fibrillation the potential capacity for prompt recovery decreases progressively. The manner of recovery is determined in part by the vigor of the rhythmic beats reëstablished, but also by the type of cardiac rhythm which develops, the degree of venous congestion and the intensity of the peripheral vasoconstriction induced by the period of general asphyxia. As shown in the upper graph of Fig. 1, a short period of asphyxia itself elevates systolic and diastolic blood pressures and increases the pulse pressure. Consequently, if fibrillation does not persist long enough to weaken the contractile power of the myocardium, such an asphyxial effect is reflected in the recovery of blood pressure. This is evident in the fibrillations lasting 11, 18 and 33 seconds in Fig. 1.

If fibrillation is prolonged—roughly more than ½ minute—this initial overcompensation in blood pressure is replaced by a slow progressive rise. For 20 to 40 seconds the heart struggles along with slow beats. As the coronary circulation and vigor of contractions gradually improve, the pressures reach supernormal levels which gradually fade back to normal ranges.

If fibrillation lasts longer—roughly a minute or more—even such a slow recovery may fail. After 2 minutes of fibrillation, regular beats are started; but under the conditions of our experiments they never become strong enough to give the myocardium sufficient nourishment for revival. Blood pressures remain low and death supervenes.

Conclusions. The dog's ventricles did not generally survive interruption of coronary circulation during fibrillation for more than about 1 minute, in the sense that useful coördinated beats sufficient for recovery are reëstablished without massage. These results caution us not to entertain too high hopes of resuscitating human hearts by the countershock method. Countershock can stop fibrillation; but complete anoxia beyond one minute generally prevents a resumption of beats sufficiently vigorous to reëstablish viable blood pressures.

11682

Antihemorrhagic Compounds as Growth Factors for the Johne's Bacillus.*

D. W. WOOLLEY AND JANET R. MCCARTER. (Introduced by O. T. Avery.)

From the Departments of Biochemistry and Agricultural Bacteriology, University of Wisconsin, Madison.

One of the first bacterial growth factors to be studied in any detail was a substance necessary for the growth of the Johne's bacillus *(Mycobacterium paratuberculosis).* Twort and Ingram[1] showed that certain other acid-fast bacteria including the tubercle bacillus contained a necessary growth factor for the original cultivation of the Johne's bacillus. Crude concentrates of the active substance were prepared from *Mycobacterium phlei.* We have long been interested in the growth of the Johne's bacillus, and for some time have attempted to isolate the active growth factor. In this paper concentration of the active factor will be described and it will be shown that it can be replaced by the antihemorrhagic vitamins.

---

* This work was supported in part by a grant from the Wisconsin Alumni Research Foundation and is published with the permission of the Director of the Wisconsin Agricultural Experiment Station.

[1] Twort, F. W., and Ingram, G. L. Y., *Johne's Disease,* London, 1913.

The strain of the Johne's bacillus grows as a dull, brittle, granular film on the surface of liquid media. It always grows better in the presence of phlei cells or an extract of them, and we have not been able to subculture it more than 2 or 3 times on a purely synthetic medium although it has been artificially cultivated about 20 years. In our experience most strains of the Johne's bacillus become continuously cultivable on a synthetic medium, although the phlei factor is needed for primary cultivation; but all strains always grow faster and give a better yield when the factor is present.

The basal medium was composed of the following substances per liter of solution: glycerol 70 cc, asparagine 5 g, sodium citrate 0.5 g, K_2HPO_4 1 g, $MgSO_4.7\ H_2O$ 1 g, iron citrate 0.063 g. Twenty cc portions of this basal medium were sterilized by autoclaving in 50 cc Erlenmeyer flasks. Substances to be tested for growth factor activity were incorporated in the basal medium at suitable dilutions. Triplicate flasks of each dilution of a substance to be tested were employed. An inoculum about 2 to 3 mm in diameter was floated on the surface of the media. The inocula could not be strictly uniform. If the cells were suspended in order to obtain uniform inocula, the lag phase of growth of the submerged cells was considerably lengthened. The bacteria for the inocula were grown on the basal medium to which had been added 2% of dried cells of *M. phlei.*

Three months were required for all the cultures to attain maximum growth; at the end of the growth period the cells were filtered from the medium, washed, dried, and weighed.

The source of the growth factor was the dried cells of *M. phlei* which had been grown on a synthetic medium.[2] One percent of this material when added to the synthetic basal medium gave excellent growth. It was found that the active substance could be extracted from the cells by boiling water, or by boiling acetone. The best concentrate obtained in this work was prepared as follows: *M. phlei* cells were extracted 3 times with boiling acetone. The extracts were concentrated under reduced pressure to an oil which was dissolved in ether. The ether solution was extracted 4 times with water. While the ether phase still contained some activity, the aqueous extracts showed maximum effect when added at a level equivalent to 1% of *M. phlei* and this amount of material supplied only 10 γ of solids per cc of medium.

---

[2] Ingraham, M. A., and Steenbock, H., *Biochem. J.*, 1935, **29**, 2553.

At this point in our studies, the note of Almquist and Klose[3] appeared, in which it was reported that phthiocol possessed vitamin K activity. Since this substance had been isolated from acid-fast bacteria[4] and since the Johne's bacillus growth factor was soluble in fat solvents as well as in water, it seemed possible that phthiocol was the active substance present in our concentrates. Dr. R. J. Anderson kindly supplied us with a sample of synthetic phthiocol, and assay showed this material to be effective in promoting growth of the Johne's bacillus. It then became of interest to test other antihemorrhagic compounds. A highly potent concentrate of vitamin K was effective, as was 2-methyl naphthoquinone (kindly supplied by Dr. A. Black of E. R. Squibb Company). Representative data are shown in Table I.

It soon became evident that while the antihemorrhagic vitamins were growth factors for the Johne's bacillus, something else in addition was supplied by the dried cells or metabolites of *M. phlei*. The growth on the phlei was more rapid as shown by the accompanying photograph (Fig. 1) taken of cultures 2 months old. Furthermore, growth was not always obtained in subcultures on the basal medium supplemented with the vitamin.

TABLE I.
Growth of the Johne's Bacillus in Media Containing Antihemorrhagic Compounds.

Culture medium	Dry wt of Johne's bacilli produced per 20 cc of medium after 3 mo growth, mg
Assay 1	
Synthetic Medium	120
" " + dried phlei cells (40 mg per cc)	260
" " + phthiocol (10 γ per cc)	207
" " + " (1.0 " ")	250
" " + " (0.1 " ")	227
" " + " (0.01 " ")	147
Assay 2	
Synthetic Medium	139
" " + phlei filtrate* (0.05 cc per cc)	195
" " + 2-methyl naphthoquinone (1.0 γ per cc)	167
" " + " " (0.15 " ")	206
" " + " " (0.1 " ")	212
" " + " " (0.01 " ")	152

* The filtrate of a culture of *M. phlei* on the synthetic medium; it had been concentrated by heat to about 1/30 the volume of the original medium. The filtrate was used instead of phlei cells here to obviate the difficulty of separating the Johne's bacilli from the phlei cells to obtain the weight of the former.

---

[3] Almquist, H. J., and Klose, A. A., *J. Am. Chem. Soc.*, 1939, **61**, 1611.

[4] Newman, M. S., Crowder, J. A., and Anderson, R. J., *J. Biol. Chem.*, 1934, **105**, 279.

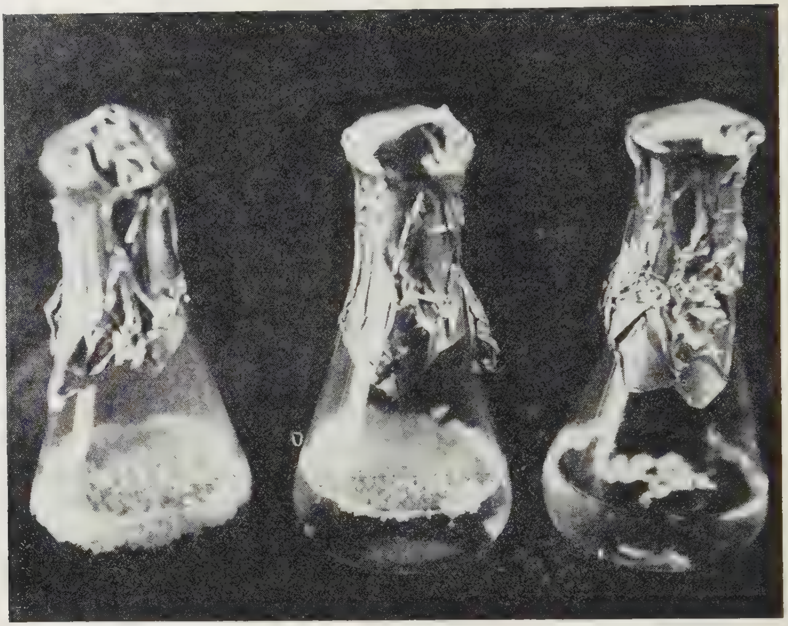


FIG. 1.

Relative stimulation of the Johne's bacillus by phlei filtrate and by 2-methyl naphthoquinone (2 months' growth).

Left—Synthetic medium + phlei filtrate.
Center—Synthetic medium + 2-methyl naphthoquinone.
Right—Synthetic medium.

Almost without exception bacterial growth factors have been found to be water-soluble compounds. Results of the present work show that the fat-soluble vitamins also play a rôle in the metabolism of certain bacteria. The effectiveness of antihemorrhagic compounds in promoting the growth of the Johne's bacillus demonstrates for the first time the part played by such compounds in bacterial nutrition. It is of interest to consider whether the utilization of a fat-soluble growth factor is in any way concerned with the very slow growth rate, or with the high fat content of these organisms. It is possible that further study with bacteria may be useful in the elucidation of the rôle of vitamin K in cell physiology.

11683

A Note on the Interrelationship of Deficiency Diseases and Resistance to Infection.*

JACKSON W. RIDDLE, TOM D. SPIES AND N. PAUL HUDSON.

From the Department of Bacteriology, The Ohio State University, Columbus; Department of Internal Medicine, University of Cincinnati; and Department of Preventive Medicine, University of Texas, Galveston.

Clinical recognition that deficiency diseases predispose to infections and, conversely, that infectious diseases predispose to vitamin deficiencies[1] suggested to us the need for a special investigation concerning this interrelationship. Although the general studies along this line are still in progress, the following observations seem sufficiently well defined to warrant publication.

Materials and Methods. The present studies were made on 150 selected cases of clinical pellagra, riboflavin deficiency and beriberi, along with other associated deficiency diseases. Before, during and after treatment, the bacterial flora of the lesions associated with the various vitamin deficiencies was investigated, venous blood for complement titrations was drawn, and the bactericidal power of the whole blood for *Staphylococcus aureus*, *Streptococcus viridans*, and *Neisseria intracellularis* was measured.

Smears from lesions and ulcers were examined and the organisms obtained by swabbing these loci were cultured and identified.

Blood for complement titrations was drawn aseptically in 3-5 cc amounts, allowed to clot on a slant in a sterile tube, and placed in the refrigerator for 18 to 24 hours. The serum was then diluted with physiological solution of sodium chloride in proportions of 1:30. Varying quantities of this dilution of complement serum decreasing from 1.0 cc to 0.1 cc, in units of 0.1 cc, were used in the titration, along with 2 units of a standardized anti-sheep hemolysin, a 2% suspension of sheep red corpuscles, and the amount of physiological saline required to bring the contents of each tube to a volume of 2.5 cc. The titrations were read after being incubated for one hour in a water bath of 38°C.

Using the Solis-Cohen technic,[2] the bactericidal power of the

---

* University of Cincinnati Studies in Nutrition at the Hillman Hospital, Birmingham, Alabama. These studies were aided by grants from the S. M. A. Corporation, Chagrin Falls, Ohio; and the National Foundation for Infantile Paralysis, Inc., New York City.

[1] Spies, T. D., *Pellagra* (Section on Deficiency Diseases), A Textbook of Medicine, Edited by R. L. Cecil, W. B. Saunders Company, 1940.

whole blood for *Staphylococcus aureus, Streptococcus viridans,* and *Neisseria intracellularis* was determined. Twenty-four-hour cultures in 20% serum dextrose broth were used in the following concentrations: undiluted, 1:5, 1:25, 1:125, 1:525; sterile 20% serum dextrose broth was used as the diluent.

Observations and Discussion. In all cases Gram-positive cocci with hemolytic properties were the dominant organisms in the lesions in the angles of the mouth associated with a deficiency of riboflavin. Eighty percent of the cases yielded pure or nearly pure cultures of hemolytic strains of *Staphylococcus aureus,* and in the remaining 20% *Streptococcus hemolyticus* predominated. Striking zones of hemolysis, frequently having a radius of 4.0 to 5.0 mm, were produced by the various strains of *Staphylococcus aureus.* Following the administration of riboflavin, or substances containing it, the lesions healed rapidly[3-7] and the organisms were no longer demonstrable. *Streptococcus hemolyticus* or hemolytic strains of *Staphylococcus aureus* dominated the bacterial flora of the noses and throats of these same patients.[8]

In association with Dr. K. W. Ascher,[9] the authors have cultured hemolytic strains of *Staphylococcus aureus* from the bacterial flora of the eye, in 14 of 30 cases of conjunctivitis occurring in vitamin deficient patients. Likewise, *Corynebacterium xerosis* in large numbers was found in nearly all of the cases, and in a pure state in the spots of Bitot, which occurred in 5 of the 30 patients.

Observers[5, 10] have found masses of Vincent's organisms in the ulcerations, so frequently seen in persons with dietary deficiency diseases, of the tongue, gums, or buccal mucosa. In addition to the heavy permeation by Vincent's organisms, 64% of these ulcerations yielded pure or nearly pure cultures of *Streptococcus hemolyticus.* In the remaining 36% of the cases, *Staphylococcus aureus,* most of the strains of which were hemolytic, was found to be the dominant organism. Within 48 hours following treatment with such therapeutic agents as nicotinic acid, cozymase, muscle adenylic acid, yeast adenylic acid, or substances, such as yeast or liver extracts, which

---

[2] Heist, G. D., Solis-Cohen, S., and Solis-Cohen, M., *J. Immunol.*, 1918, **3**, 261.

[3] Sebrell, W. H., and Butler, R. E., *Public Health Rep.*, 1938, **53**, 22.

[4] Sebrell, W. H., and Butler, R. E., *Public Health Rep.*, 1939, **54**, 2121.

[5] Spies, T. D., Bean, W. B., and Ashe, W. F., *Ann. Int. Med.*, 1939, **12**, 1830.

[6] Spies, T. D., Vilter, R. W., and Ashe, W. F., *J. Am. Med. Assn.*, 1939, **113**, 931.

[7] Vilter, R. W., Vilter, S. P., and Spies, T. D., *J. Am. Med. Assn.*, 1939, **112**, 420.

[8] Riddle, J. W., Spies, T. D., and Hudson, N. P., unpublished observations.

[9] Riddle, J. W., Ascher, K. W., Spies, T. D., and Hudson, N. P., unpublished observations.

[10] Spies, T. D., and Cooper, C., *International Clinics*, 1938, Vol. 4, Series 47.

are rich in these factors, these ulcerated areas decreased in size, healed,[11] and the organisms disappeared.

From three hundred and seventy-five complement titrations, the following observations and interpretations have been made: the acutely deficient persons studied, with notably few exceptions, have a surprisingly low amount of complement. In persons with advanced deficiency disease, hemolysis was barely detectable in the tube containing the largest amount of complement serum (1.0 cc), in contrast to the average normal complement titre which produced a one plus hemolysis in the tube containing 0.4 cc of 1:30 complement serum. Following the administration of therapeutic agents, the complement titre increased along with the clinical improvement of the patient to a normal level within one to 5 days. The blood from subclinical and mild pellagrins contained either a normal or slightly subnormal titre which increased following treatment for the deficiency.

We find a pronounced depression in the bactericidal power of the whole blood for *Staphylococcus aureus* in the patients suffering more acutely from a deficiency of the Vitamin B complex. The blood of subclinical and mild pellagrins shows only a slight variation below that of our normals in its staphylococcidal power. The titres of sensitizing antibodies for *Streptococcus viridans* and *Neisseria intracellularis* were little affected by the presence of a Vitamin B complex deficiency. Findlay and MacLean[12] noted that rats, when maintained on a "Vitamin B free diet", exhibited a drop in the bactericidal power of the whole blood for *Staphylococcus aureus,* while the titres of natural antibodies for other bacteria were apparently uninfluenced by this deficiency.

Summary and Conclusions. 1. Our observations in 150 patients from a region of Alabama in which deficiency diseases are endemic show a relationship between these diseases and the resistance to, and presence of, infections with *Staphylococcus aureus* and *Streptococcus hemolyticus.* 2. The lesions at the corners of the mouth, characteristic of riboflavin deficiency, contained pure or nearly pure cultures of hemolytic strains of *Staphylococcus aureus* in 80% of the cases, and in the remaining 20%, *Streptococcus hemolyticus* predominated. Following the oral or intravenous administration of riboflavin or substances rich in it, the fissures healed rapidly and the organisms were no longer demonstrable. 3. When the bacterial flora of the conjunctival sacs were studied in cases of dietary deficiency disease and associated conjunctivitis, hemolytic strains of *Staphylococcus*

---

[11] Spies, T. D., Vilter, R. W., and Bean, W. B., unpublished observations.

[12] Findlay, G. M., and MacLean, I. S., *J. Biochem.*, 1925, **19**, 63.

aureus were found to predominate in 14 of the 30 cases. Smears and cultures demonstrated the presence of *Corynebacterium xerosis* in a pure state in all of the spots of Bitot which occurred in 5 cases. 4. In addition to masses of Vincent's organisms, 64% of the ulcerations of the tongue, gums, or buccal mucosa yielded *Streptococcus hemolyticus,* and the remaining 36% contained hemolytic strains of *Staphylococcus aureus.* Following specific therapy with anti-pellagric substances, the bacterial flora of these ulcerations, including the Vincent's organisms, promptly disappeared.

5. A low complement titre exists in acutely deficient patients, and in the subclinical and mild cases the titre is slightly subnormal or normal. Following clinical improvement the complement titre increases. 6. In the whole blood of acutely deficient patients there is a distinct depression in the bactericidal power for *Staphylococcus aureus,* whereas, only a slight diminution in staphylococcidal power was observed in the blood of subclinical and mild cases of vitamin deficiency.

11684 P

Attempts to Produce Poliomyelitis in Eastern Cotton Rats with Flexner's M. V. Strain.*

JOHN A. TOOMEY AND WILLIAM S. TAKACS.

From the Division of Contagious Diseases, City Hospital, Cleveland, O., and the Department of Pediatrics, Western Reserve University.

Forty-five cotton rats were injected with 9 strains of poliomyelitis virus (Flexner M. V., Flexner W. E., Toomey, Philadelphia, Kramer, Harmon, Howitt, Trask, W. E., Australian). The doses injected were the same as those used by Armstrong[1] to produce poliomyelitis, *i. e.,* 0.06 cc intranasally, 0.06 cc intracerebrally and 0.5 cc subcutaneously of a 10% cord brain suspension. After 6 weeks of observation, 11 animals had died—2 after the injection of Harmon's and one each after the injection of the other strains, except Toomey's and Trask's. The cords and brains of those animals that died were made up into 10% suspensions in saline and injected into other animals. None of the latter either died or developed paralysis even after 4 months of observation.

---

* Aided by a grant from the National Foundation for Infantile Paralysis, Inc.

[1] Armstrong, C., *Public Health Rep.*, 1939, **54**, 1719.

Flexner's M.V. virus strain, which was also our most potent monkey poliomyelitis virus strain and the one most recently passaged by us, was injected into 25 other cotton rats in the same manner as described before. The results were negative.

Previously, it was found that the addition of 10 day colon typhoid paratyphoid (list of organisms used,[2]) toxic broth filtrate (called "enteric organism filtrate" for convenience) to poliomyelitis virus accelerated the production of the disease when the combination was injected intracerebrally into monkeys.[3] Perhaps such a virus filtrate mixture would produce poliomyelitis in Eastern cotton rats. Ten per cent virus suspensions in enteric organism filtrate were used throughout save where otherwise noted.

Ten animals were injected with the standard dose in enteric organism filtrate in the usual way. Three animals were living after 4 months. The 2 which died of shock on the first day and 2 which died on the sixty-ninth day were discarded. One animal died the ninth, one the twelfth and one the eighteenth day after injection without developing paralysis. They merely became furred and lost weight. A suspension of the cords and brains of the latter animals in enteric organism filtrate was injected into 5 animals, only 1 of which died on the eleventh day without showing any paralysis. In turn, a suspension of its cord and brain was injected into a third generation of 5 animals, one of which died without paralysis. A suspension of its cord and brain was injected into a fourth generation of 5 animals. In an attempt to break down the resistance of these animals, they were given 4 additional subcutaneous injections of the virus-toxin combination—0.5 cc every third day. There was a marked weight loss; all 5 animals died, but none had paralysis. A combination of their cords and brains was made up for 5 animals in the fifth generation. Only the first injection was given for all of these animals died and all but one developed typical paralysis. The latter might have had paralysis for it became extremely weak and ataxic during the day. No definite assertion could be made, however, since it died during the night and was not observed. The brains and cords of these animals were combined and passaged to 3 animals of the sixth generation. All the rats developed paralysis and died. It was then decided to see if the strain had become acclimated to the rat and saline was used as the diluent for the virus suspensions injected into the 2 rats of the seventh generation. Both animals developed paralysis. The incubation period was longer, however, with saline than when enteric toxin filtrate was used as the diluent.

---

[2] Toomey, J. A., *J. Inf. Dis.*, 1934, **54**, 74.

[3] Toomey, J. A., PROC. SOC. EXP. BIOL. AND MED., 1934, **31**, 1015.

Saline diluted virus suspension of the seventh was injected into 2 animals of the eighth generation. Both developed paralysis and died. Three animals of the ninth injected with saline suspension of the eighth generation developed paralysis and died.

The virus has been passed from rat to *M. malatta* monkey in the sixth and eighth generation, respectively, and the disease produced. It has also been passed from monkey to monkey, but not as yet from monkey subpassage back to rat.

Differences are found in Eastern cotton rats that have been injected with Jungeblut's acclimated Trask virus strain,[4] the Armstrong and our own acclimated Flexner's M.V. strain. When saline was used as the diluent for virus suspension, the incubation period was about the same for Jungeblut's as for Armstrong's virus strain. The animals started to come down within from 3 to 4 days, becoming furred and weak at first and dying quickly—possibly from respiratory paralysis before they became completely quadriplegic. Often the animals were found dead with only a brief period between objective symptoms of furring, incoördination and death. On the other hand, it took from 6 to 7 days for the animals injected with Flexner's strain to become ill, the rats generally becoming paralyzed in the hind quarters about the sixth to the eighth day and though the paralysis spread quickly enough, it was comparatively slower than after the use of the other strains. The animals injected with Flexner's strain usually became furred after or at the same time paralysis set in, but not before.

Experiments are now in progress to determine whether other strains of virus will respond in a similar fashion.

---

[4] Jungeblut, C., and Sanders, M., PROC. SOC. EXP. BIOL. AND MED., 1940, **44**, 375.

11685

Protection of Chick Against Rous Sarcoma Virus by Serum from Adult Chickens.*

F. DURAN-REYNALS AND E. ESTRADA.

From the Department of Bacteriology, Yale University School of Medicine.

Paralleling the growth and differentiation of fowls, antibody-like factors against many antigens appear in succession in their blood with remarkable regularity.[1] The blood of new hatched chicks is entirely devoid of such factors whereas the blood of practically all adult chickens contains them. Viruses inducing tumors in fowls, such as the Rous and Fuginami sarcomas and Mill Hill *2* endothelioma, are likewise strongly suppressed by the sera of most adult chickens, but the Rous virus—the only one studied in this respect—is only occasionally suppressed by the blood serum of chicks.[2] Newly hatched chicks injected intravenously with the Rous and Fuginami viruses constantly developed a fatal disease characterized by hemorrhagic lesions (blebs and diffuse extravasations) in the absence of gross neoplastic lesions; pullets developed a disease of a combined sort where hemorrhagic and neoplastic lesions were observed side by side; adult chickens developed a disease less frequently, and when present, consisted in most cases of lesions of the usual neoplastic type.[3] The latter results confirmed observations of previous workers on the subject.[4] No final conclusions were reached as to the action of the sarcoma viruses on the vascular system of the chick. The fact that histological study of the earliest hemorrhagic lesions often failed to disclose the presence of tumor cells was suggestive of an effect of the virus other than neoplastic, but the conservative explanation that hemorrhages were, after all, the result of minute mural or intravascular growth could not be discarded on the evidence available.

When the Rous virus was injected into tissues of chickens of different ages the incidence and rate of growth of tumors was higher in chicks than in pullets, and higher in pullets than in adult chickens.[2, 5] In the *2* former hosts lesions in the inoculated tissues were

---

* This investigation was aided by a grant from the Jane Coffin Childs Memorial Fund for Medical Research.

[1] Duran-Reynals, F., *Yale J. Med. and Biol.*, 1940, **12**, 361.

[2] Duran-Reynals, F., *Yale J. Med. and Biol.*, 1940, **13**, 61.

[3] Duran-Reynals, F., *Yale J. Med. and Biol.*, 1940, **13**, 77.

[4] Rous, P., Murphy, J. B., and Tyttler, W. H., *J. Am. Med. Assn.*, 1912, **58**, 1751; Doerr, R., Bleyer, L., and Schmidt, *Z. f. Krebsforsch.*, 1932, **36**, 256.

[5] Rous, P., and Murphy, J. B., *J. Exp. Med.*, 1914, **20**, 419.

often hemorrhagic, and practically all of them developed metastatic lesions both neoplastic and hemorrhagic. Some of the latter were presumably induced by virus liberated from the primary lesion. Summarizing: In chicks and pullets, hosts with blood devoid of neutralizing power for the Rous virus, certain cells of the vascular system are attacked by the virus with the production of hemorrhagic lesions, and tumors grow very fast in their tissues. On the contrary in adult chickens, hosts with blood possessing a strong neutralizing power for the Rous virus, the cells of the vascular system are either insusceptible or protected against the virus, and tumors grow comparatively slowly in their tissues. Moreover,[2] in the latter hosts, the pronounced individual differences in the size of tumors following inoculations of virus in their tissues were shown to be in an inverse relation with the neutralizing power of the blood for the virus.

Experimental: Chicken serum was procured from normal Plymouth Rocks 8 months old, the serum from 10 chickens being pooled in aliquots. As a source of Rous virus fresh tumor tissue was extracted with 19 parts of saline solution, and the extract passed through a Berkefeld N candle. In general the experimental procedure was as follows: new hatched Plymouth Rock chicks were first injected in the peritoneal cavity with 0.5 cc of chicken serum and, as a control, as many chicks were similarly injected with either saline solution or rabbit serum. Next day the chicks were injected in the jugular vein with 0.5 cc of tumor filtrate, and the peritoneal injections of sera or saline were repeated daily until the first chick died, usually from 8 to 12 days after the injection; then the rest of the animals were sacrificed and studied. If none of the chicks had died before 12 days they were likewise sacrificed. Departures from this technique will be described as the occasion arises.

Out of 12 experiments thus carried out protection against the effects of the virus by treatment with chicken serum were obtained in 9. These comprise a total of 78 treated chicks and 80 controls. In Table I results are grouped according to: (a) Incidence and type of

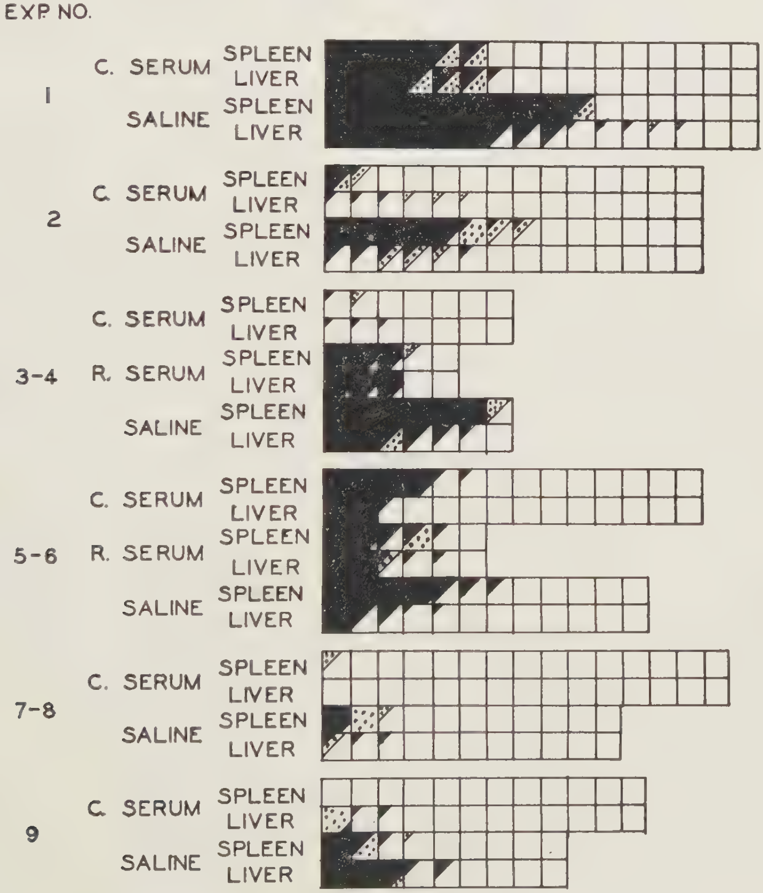
TABLE I.
Protection of Chicks Against the Effects of the Rous Virus by Treatment with Serum from Adult Chickens.

Treatment	Total	Lesions around injected vein	Generalized lesions: Hemorrhagic	Generalized lesions: Combined	Generalized lesions: Neoplastic	Blood clot in peritoneum	Death within 12 days
Chicken serum	78	18*	19	11	4	9	0
Rabbit serum or saline sol.	80	41†	43	18	1	26	17

*All neoplastic.
†6 hemorrhagic, 35 neoplastic.

gross local lesions, (b) incidence and type of gross generalized lesions, and (c) incidence of death within 12 days following the virus injection. These results will be analysed separately.

(a) *Local Lesions.* The lesions which develop around the injected jugular vein are the result of soiling the tissues by minute amounts of virus. As described elsewhere[3] they are of 3 sorts: purely hemorrhagic (blebs); combined hemorrhagic—neoplastic; and purely neoplastic. Besides the differences indicated in Table I, the tumors in the treated group were smaller than those of the control group; in either group they had at best an area of 1 sq cm. In a more direct study of the influence of the treatment upon occurrence of local tumors, the following experiments were carried out.



TEXT-FIGURE 1.

Each square represents one chick; a wholly black on dotted square indicates an organ studded with hemorrhagic and neoplastic lesions respectively while lesser degrees of hemorrhagic or neoplastic involvement are indicated by correspondingly smaller black or dotted fractions of the square. C—chicken; R—rabbit.

Experiments. In 3 different investigations 52 chicks in 2 groups were injected intradermally in the breast with 0.5 cc of a 1:200 dilution of filtrate of Rous tumor. Half the animals were treated daily with chicken serum and the other half with saline solution, as above described, for 20 days. Tumors resulted in most cases, and they were of the same size in both groups. The study was repeated on 14 chicks with the only difference that they were given 8 daily peritoneal in-

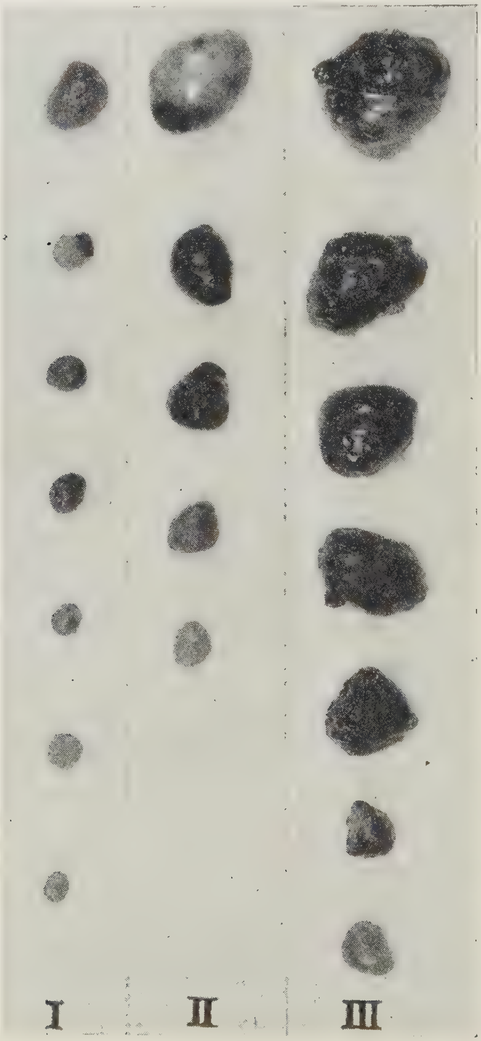


PLATE 1.

Experiments Nos. 3 and 4 in Text-fig. 1. Spleens from chicks treated with (I) normal chicken serum, (II) normal rabbit serum, and (III) saline solution.

jections of 0.5 cc of chicken serum before and 25 after the virus was injected intradermally. Results were as follows: 50% of the chicks in the treated group and 88% in the control group died of their tumors, the average areas of the tumors being 5.5 and 12 sq cm respectively. The effect of the treatment on the incidence and type of metastatic lesions will be taken up in the following section.

(b) *Generalized lesions.* These had been fully described elsewhere.[3] Since the organs constantly affected are the spleen and liver, the differences in their involvement, expressed graphically in text-figure I, and Plate I can be taken as a faithful index of the intensity of the infection.

Moreover, 12, 10 and 3 chicks in the treated group against 21, 25 and 16 in the control group showed lesions in the lungs, gonads and intestines respectively; in gonads and intestines they were hemorrhagic (blebs) in both groups; in the lung (diffuse extravasations) were always hemorrhagic in the control group, but sometimes neoplastic in the treated group. Study of the chicks that died of their skin-induced tumors, in the foregoing section, showed that treatment with chicken serum also afforded a protection against or changed the type of the metastatic lesions. In the treated group the lungs were replaced by tumor, the livers showed equal proportions of tumors and blebs, but the spleens were free of disease. In the control group the lungs—always neoplastic—showed also extensive extravasations of blood, and the livers, and also the spleens, showed exclusively hemorrhagic lesions.

In the 3 experiments where no protection against the virus injected intravenously was observed the tumor preparations employed were unusually active. In another experiment a single dose of serum (5 cc) was injected intraperitoneally shortly after a virus preparation of medium activity was injected in the vein. No protection was obtained.†

(c) *Time of death.* Longer survival in the treated group is clearly suggested, in Table I, by the fact that all the 17 chicks that died belonged to the control group. The fact was directly shown by an experiment (9 in text-fig. I) where chicks were given 8 daily peritoneal injections of 0.5 cc of chicken serum preceding, and 20 more following the intravenous injection of 0.5 cc of tumor filtrate at 1:200. The control chicks were similarly injected with saline solution. In the treated group 3 of the 12 chicks died 25, 36 and 38 days after the virus injection, and when the remaining chicks were killed 50 days after this injection, no lesions were found in them. In the control group 5 out of the 9 chicks died 10, 15, 16, and 20 days after injection of virus, and in 2 of the remaining 4, killed after 50 days, actively growing tumors were found.

---

† This particular experiment can, in a way, be compared to that carried out by Rous and Murphy[5] in which from 35 to 65 cc of blood from immune fowls were transfused in one time to 5 fowls bearing Rous tumors, and the growths were not affected. Both experiments pose the questions whether the disease in the adult could be controlled by daily injections of serum as the disease in the chick is; presumably the experiment, if attempted, would require almost prohibitive amounts of serum.

Discussion and Summary. Whatever vascular cells are affected, the induction of hemorrhagic lesions by the Rous virus indicates extreme susceptibility such as that usually found in the chick.[3] The present results show that adequate treatment of such hosts by serum from adult chickens—presumably through the antibody-like factor there present—causes either a shifting from hemorrhagic into neoplastic lesions or suppression of all lesions. In other words the treated chicks react to the virus much as adult chickens do. This goes to show that in fowls the age differences in the response to the Rous virus are dependent upon factors in the blood, and confirms the supposition that the relative ineffectiveness of the virus upon the vascular system of the adult is due to the selective protection by the blood of the susceptible cells of the vascular wall whereas the susceptible extravascular cells are less protected. Presumably these cells in the chick are still less protected since after an injection of virus, large tumors always arise from them.[2] The present results show that treatment with adult's serum is also effective in protecting these extravascular cells as long as the virus has been injected in the tissues in very small amounts, as presumably happens in the procedure of the intravenous injection. or when the chicks have been prepared for several days before larger amounts of virus were injected intradermally.

11686

Effect of Gastric Juice on Erythrocytes of Newborn Rats.

T. A. Noble, H. S. Wigodsky and A. C. Ivy.

From the Department of Physiology and Pharmacology, Northwestern University Medical School, Chicago.

There is no known condition in animals which resembles pernicious anemia in man. However, the fetuses and newborn of some animals have a macrocytic anemia (in comparison to adult values) which resembles to some extent the hematologic conditions found in pernicious anemia. This is especially true of the rat. If, through the use of materials effective in treating pernicious anemia, this hematologic picture could be converted into one resembling the adult, the newborn rat might provide a convenient method for the assay of anti-pernicious anemia substances.

Several reports might be cited in support of this possibility. The administration to the pregnant female rat of ventriculin,[1, 2] normal human and hog gastric juice,[3, 4] gastric juice from patients with achlorhydria and gastric carcinoma[5] has been shown to reduce the macrocytosis of the newborn rat. Gastric juice from pernicious anemia patients is relatively ineffective in this regard.[5] Liver extract injected into immature opposums for three to fourteen days while they were in the maternal pouch has been demonstrated to markedly affect the hematologic picture.[6]

On the other hand the possibility of using newborn rats is not supported by the results previously reported by us[7] and confirmed by Bruner.[8] A liver extract known to contain the antipernicious anemia factor did not affect the blood picture of the newborn rats of mothers receiving the treatment during pregnancy.

In view of these conflicting reports, we have repeated the work of Stasney and Higgins attempting to affect the macrocytosis of newborn rats by administering normal human gastric juice parenterally and orally to the pregnant mother.

---

[1] Briese, E., and Higgins, G. M., *Anat. Rec.*, 1939, **73**, 105.

[2] Jones, O. P., *Anat. Rec.*, 1940, **76**, 34.

[3] Stasney, J., and Higgins, G. M., *Proc. Staff Meet. Mayo Clinic*, 1937, **12**, 490.

[4] Stasney, J., Higgins, G. M., and Mann, F. C., *Proc. Staff Meet. Mayo Clinic*, 1937, **12**, 399.

[5] Schlicke, C. P., *Proc. Staff Meet. Mayo Clinic*, 1939, **14**, 145.

[6] Stasney, J., and Burns, E. L., Proc. Soc. Exp. Biol. and Med., 1939, **42**, 544.

[7] Wigodsky, H. S., and Ivy, A. C., Proc. Soc. Exp. Biol. and Med., 1938, **38**, 787.

[8] Bruner, H. D., Proc. Soc. Exp. Biol. and Med., 1939, **41**, 260.

Methods. Pregnant stock rats of our albino colony were used in this experiment. Gastric juice was obtained daily from normal human subjects following the injection of 1 mg of histamine subcutaneously. In the first series, the gastric juice was concentrated 10 times *in vacuo* and 1 cc injected intraperitoneally into the pregnant rat daily for 4 to 9 days before delivery. In the second series, 3 cc of fresh normal human gastric juice was administered by stomach tube to the pregnant rat daily for 4 to 10 days before delivery.

Hematologic examinations were performed as soon after birth as possible, or within 12 hours. Blood for cell counts, hemoglobin determinations, and smears were obtained by cutting off the tail of the newborn rat. Red blood cell counts were made in the usual manner. Hemoglobin was determined by the Newcomer method. The mean red blood cell diameters were determined on dry smears stained with Wright's stain.

The number of animals used in each group is shown in the table.

Results and Discussion. The averaged results are shown in the table. Although there were some significant increases in red blood cell and hemoglobin values in the newborn rats whose mothers had received gastric juice, the increases are neither large nor proportional

TABLE I.
Erythrocyte Values of Newborn Rats Whose Mothers Received Gastric Juice.

Days treated	Method of administering gastric juice	No. Litters	No. Pups	R.B.C. $\times 10^6$/mm^3	Hemoglobin g/100 cc	Mean diameter, μ
0	Controls	20	144	2.89±0.02†	14.11±0.13	8.42±0.02
4	Injected intraperiton.*	1	5	3.03±0.09	14.61±0.25	8.11±0.18
5	” ”	3	18	2.93±0.05	14.27±0.06	8.35±0.04
Avg	4 to 5 days	4	23	2.95	14.35	8.30
6	Injected intraperiton.	3	22	3.10±0.07	14.17±0.21	8.33±0.05
7	” ”	3	20	2.72±0.05	12.93±0.22	8.51±0.03
9	” ”	1	6	3.36±0.09	15.96±0.32	8.25±0.06
Avg	6 to 9 days	7	48	2.91	13.88	8.40
4	Stomach tube‡	1	9	3.51±0.07	13.70±0.15	8.52±0.03
5	” ”	3	18	3.14±0.07	13.71±0.27	8.52±0.03
Avg	4 to 5 days	4	27	3.27	13.70	8.52
6	Stomach tube	4	23	3.07±0.04	15.96±0.29	8.53±0.02
7	” ”	1	5	3.26±0.15	15.70±0.50	8.45±0.03
8	” ”	2	20	3.37±0.06	15.23±0.13	8.43±0.03
9	” ”	3	17	3.19±0.06	13.44±0.20	8.35±0.02
10	” ”	1	9	3.50±0.08	17.11±0.22	8.65±0.04
Avg	6 to 10 days	11	74	3.24	15.33	8.47

*1 cc daily, gastric juice concentrated 10 times *in vacuo*.
†Probable error.
‡3 cc daily normal gastric juice.

to the duration of treatment. In no instance was there a significant increase or decrease in the mean red blood cell diameters.

Stasney and Higgins reported only the largest diameters and mean corpuscular volume of the red blood cells in the newborn rats whose mothers received normal human and hog gastric juice but did not report the mean diameters. These "largest" diameters were measured from photographs of smears. The cells were selected at random and the largest diameter of the cell was measured, *i. e.,* if the cell was oval, the long axis was used rather than the short. This method appears to us to be too arbitrary to be used in determining the effect of anti-pernicious anemia material.

Conclusions. The administration of normal human gastric juice to pregnant rats by intraperitoneal injections of concentrated juice and by stomach tube of unconcentrated juice was without effect on the mean red blood cell diameters of the newborn rats.

11687 P

Cultivation of Relapsing Fever Spirochetes in Embryonic Chick.

S. W. BOHLS, J. V. IRONS AND THELMA DE SHAZO.
(Introduced by C. W. Duval.)

From the Division of Laboratories, Texas State Health Department.

Since its use for the cultivation of a spirillum of fowls by Levaditi[1] and the fowl-pox virus by Woodruff and Goodpasture,[2] the fertile hen egg has been employed in the cultivation of many viruses. This medium was employed by Cox[3] for the cultivation of Rickettsiae, and has also been utilized in the study of bacteria[4] and protozoa.[5] Morrow, Syverton and their collaborators[6] employed the developing hen egg in the study of *Leptospira icterohemorrhagiae,* but Bessemans and De Meirsman[7] failed with *Treponema pallida.* Employing murine-passaged strains, both Oag[8] and Chabaud[9] were successful with *Borrelia duttoni*.

---

[1] Levaditi, C., *Ann. Inst. Past.*, 1906, **20**, 924.

[2] Woodruff, A. M., and Goodpasture, E. W., *Am. J. Path.*, 1931, **7**, 209.

[3] Cox, H. R., *Pub. Health Rep.*, 1938, **53**, 2241.

[4] Goodpasture, E. W., and Anderson, K., *Am. J. Path.*, 1937, **13**, 149.

[5] Longley, B. J., Clausen, N. M., and Tatum, A. L., PROC. SOC. EXP. BIOL. AND MED., 1939, **41**, 365.

[6] Morrow, G., Syverton, J. T., Stiles, W. W., and Berry, G. P., *Science*, 1938, **88**, 384.

[7] Bessemans, A., and De Meirsman, E., *C. R. Soc. biol.*, 1938, **127**, 847.

Owing to the difficulty of cultivating spirochetes of relapsing fever in laboratory media, we have attempted to grow these organisms in the developing hen egg. In this communication we are reporting successful cultivation of Texas relapsing fever spirochetes in this medium with blood from each of four patients. Specimens were received in the routine mail and after Giemsa preparations of the clots were examined, each specimen was divided in two portions. The first portions of the 4 inocula were introduced intraperitoneally in rats without delay, while an additional day of delay was incurred by sterility testing of the second portions prior to the inoculation of fertile eggs. Meanwhile, previously incubated white leghorn eggs were candled and scrubbed clean with warm soap and water.

For the first inoculum 10-day-old eggs were selected and the chorio-allantoic membranes were exposed by means of a modified dental drill, for the window method of study.[2] On each of 8 chorio-allantoic membranes was dropped 0.2 cc of the specimen and the membranes were observed through the window daily for possible changes or lesions. Although chorio-allantoic lesions were not observed, beginning with the third day after inoculation, an egg was sacrificed daily for the removal of portions of chorio-allantoic membrane, yolk, amniotic fluid and embryonic blood. Portions of these tissues and fluids were examined in appropriate darkfield or Giemsa-stained preparations for possible content of spirochetes. Spirochetes were first found in blood-tinged amniotic fluid on the fifth day after inoculation. Spirochetes were not demonstrated in non-vascular portions of the chorio-allantois, amniotic fluid devoid of blood cells, or yolk.

For each of 3 subsequent primary inoculations, 9-day-old eggs were selected and the yolk-sac method was utilized.[3] Several fertile eggs, as immediately determined by candling, were placed in wine glasses or beakers with the air-sac end upwards. After the shell had been thoroughly scrubbed with alcohol a hole was tapped with the end of a heavy pointed probe and 0.2 cc of material was injected at an approximate depth of 2 cm, after which the hole was sealed with paraffin. Beginning with the third day and daily thereafter, the chorio-allantois of an egg was exposed for the collection of amniotic fluid and blood. By the 3rd to 7th day spirochetes had appeared in bloody amniotic fluid with each of the 3 inocula. In no instance were spirochetes found in any preparation which was devoid of blood. In many instances the spirochetes were found in enormous numbers, often occurring as matted masses. In some preparations they were nearly as numerous as blood cells, attesting to the suitability of the fertile egg as a culture medium.

---

[8] Oag, R. K., *J. Path. and Bact.*, 1939, **49**, 339.

[9] Chabaud, A., *Bull. Soc. Path. Exot.*, 1939, **32**, 483.

Two strains are being passaged weekly in 8-10-day-old eggs. Apparently the passages may be continued indefinitely. Five-day-old eggs have been less satisfactory than 8-10-day-old eggs. Twelve-day-old eggs also seem to be satisfactory. Since most of these eggs have been sacrificed by the sixteenth day of development, the ultimate effect of infection on embryonic life has not been determined, but spirochetes have not been found in the blood of a few embryos which survived until the hatching stage. Rats developed typical infections as a result of inoculation from the original specimens, but tests for possible alterations in virulence as a result of egg passage have not been done. Cultivation in the embryonic blood of chicks may be useful as an aid in the diagnosis of relapsing fever or for the maintenance or study of the spirochetes.

11688

Is Chromodacryorrhea a Diapedesis of the Red Corpuscles?

Shiro Tashiro, Elizabeth Badger and Waldo Younker.
From the Department of Biochemistry, College of Medicine, The University of Cincinnati, Cincinnati.

When dacryorrhetin, a substance prepared from muscle, is injected into rats, the animals shed bloody tears. This peculiar phenomenon of weeping bloody tears by rats was designated as chromodacryorrhea.[1, 2] These bloody tears come from Harder's glands without apparent injuries to the blood vessels.[3] These facts together with the amazing speed with which this phenomenon occurs and the fact that other cholinergic substances produce it raises an interesting question, namely: Is chromodacryorrhea a diapedesis of blood?

According to Freud,[4] who discovered a similar phenomenon with

---

[1] Tashiro, Shiro, and Stix, Helen, *Biol. Bull.*, 1935, **64**, 327.

[2] Tashiro, Shiro, *Proc. Am. Soc. Biochemists*, 1937, **8**, xcviii; Kongressbericht. II les XVI Internat. Physiologenkongress 1938, 46.

[3] Stix, Helen, unpublished data.

[4] Freud, J., *Acta Brevia Neerl.*, 1933, **3**, 159.

acetylcholine, and pilocarpine, the tears are tinged red with *blood* and he concludes that "substances with parasympathetic action cause a diapedesis of blood into the conjunctiva in rats". For this conclusion, as Selye[5] first pointed out, Freud does not present any evidence. In justice to Freud, however, we may state that ordinarily, for the bloody fluid coming out of the mammalian tissue, no one is expected to offer scientific proof for blood. In fact none of those who had seen our demonstration of the bloody tear flow in rats following dacryorrhetin injection, or the actual color photographs on the kodachrome film, even raised the question.

Nevertheless, we thought it would be advisable not to take anything for granted, and to see first if bloody tears contain blood; and if so, to see whether there is a transudation of erythrocytes through unruptured blood vessels.

Experimental. Collection of tears. Chromodacryorrhea is produced by intraperitoneal injections of dacryorrhetin. In later experiments, acetylcholine and carbamylcholine are also used. For microscopic examinations, the microscopic slides are directly applied over the tears, and for other experiments, the tears are pipetted off into physiological salt solutions. In either event, only the red tears alone were used for examination.

Routine blood tests. When examined under a low power microscope, one finds a large number of spherical bodies, tinged red quite similar to the red cells. The number of these bodies amounted to 500,000 per cmm in one sample from a rat, the blood of which contained 6 million erythrocytes per cmm. The saline suspension of the tears is turbid red as though the cells were not hemolysed. Such a solution shows 2 characteristic bands of α and β of oxyhemoglobin when examined casually with a hand spectroscope, and it gives a benzidine test.

In spite of the fact that the casual examinations were suggestive of the presence of red blood corpuscles in the tears, we were surprised to find that "the cells" could not be stained by ordinary blood staining methods. More careful microscopic examinations under high power revealed that these round bodies are not evenly tinged red, and the red pigment is not confined in them alone. In fact, very often the red pigment is scattered, being cramped in amorphous granular shapes outside of these spherical bodies. Besides all the bodies are not exactly the same size and here and there one finds "giant" bodies which have the appearance of hollow globules. The most outstanding difference from erythrocytes is that they lack the appearance of having

---

[5] Selye, Hans, *Canadian Med. Assn. J.*, 1937, **36**, 200.

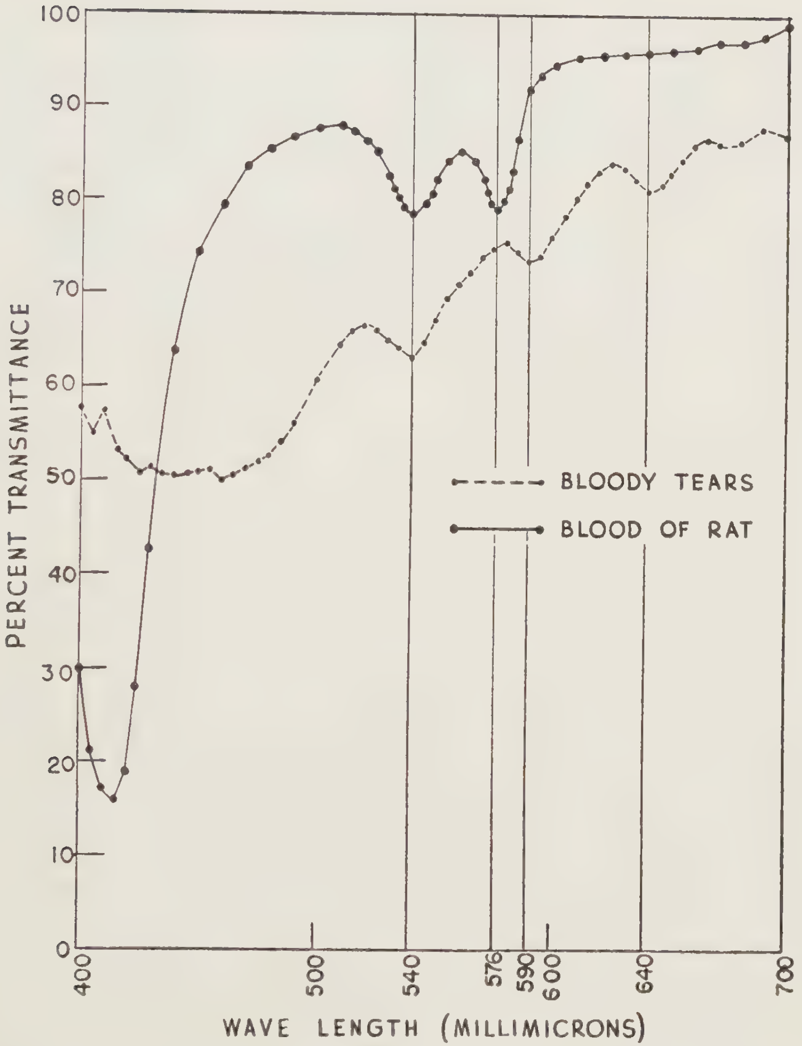


Fig. 1.

a definite cell wall. When the saline or aqueous solution (in suspension) is centrifuged, these globules are separated on the top as a colorless layer, and the red pigment is thrown down at the bottom.

The nature of the red pigment. The pigment is not very soluble in distilled water, although the solution gives a pink color. When this solution is compared with hemolysed blood with a hand spectroscope, by placing 2 spectra exactly side by side, the 2 absorbing bands are not exactly at the same position. Although the bands at β position are identical, the α of oxyhemoglobin is slightly to the right as compared with that of the bloody tear pigment.

The spectrophotometric analysis. The further analysis of the absorption spectra of the red pigment was done with a spectrophotometer by comparing it with that of rat blood (1 :500 dilution). The result is given in the figure, which shows that both have the β band at 540 millimicrons, but the tear pigment has the α band at 590, instead of the customary α band of hemoglobin at 576 millimicrons. The red pigment of the tear also has another band at 640 millimicrons, but has no band at 415 millimicrons as does the blood.

These characteristic bands can be detected with saline suspension of the tears, or supernatant fluid of the centrifuged sample, or resuspension of the pigment in water after the centrifuging.

Ultraviolet fluorescence. A further difference between the red pigment of bloody tears and hemoglobin is the fact that a solution of the red pigment of the tears gives a red fluorescence when excited by ultraviolet ray filtered through a Corex filter (90% efficiency at line 366 millimicrons).

Could chromodacryorrhetic pigment be due to the action of clear tears on hemoglobin?

We added a small amount of hemolysed blood to the clear tears of rat and examined the mixture. The results show that the absorption spectra of the mixture were identical with those of blood; and the mixture gave no ultraviolet fluorescence. Thus, the red pigment of the chromodacryorrhetic tear is not identical with the mixture of blood and clear tear, and is not a reaction product between clear tears and hemoglobin.

Conclusion. These facts definitely prove in our minds that the bloody tears contain no erythrocytes, and the red pigment in chromodacryorrhesis is not hemoglobin. The minute pink corpuscular bodies are in reality colorless fatty globules, tinged red by the pigment that surrounds them. In other words, chromodacryorrhesis is not a diapedesis of erythrocytes, and the tears do not even contain hemoglobin. It is really a most surprising fact for those who are familiar with the phenomenon of chromodacryorrhea that such obviously bloody fluid coming out of mammalian tissue does not contain any blood.

On the other hand, these findings now seem to help explain many puzzling facts. One of us (with Stix)[1] found that dacryorrhetin causes bloody tear production only in rats (with occasional exceptions with mice), milky tears in guinea pigs, rabbits and mice, and clear tears in cats and dogs. Just why rats alone should shed bloody tears has been one of the perplexing problems. On the basis of the above findings, we can safely attribute the cause of chromodacryorrhea to a peculiarity of rats in respect to Harder's glands.

Derrien and Turchini[6] describe a substance, a porphyrine, in Harder's glands of rats and in lesser amount in those of mice. One wonders if this porphyrine is identical with the bloody pigments of the tears, or gives rise to it.

Preliminary experiments (by B) suggest that these two are probably identical as judged by ultraviolet fluorescence and the solubility characteristics of pigments as well as of their salts. Just exactly what the chemical nature of this pigment in the tears is and what the function of dacryorrhesis is, is another problem.

11689 P

Preservation of Cultures of *N. gonorrhoeae*.*

LUCILE R. HAC. (Introduced by H. Close Hesseltine.)

From the Department of Obstetrics and Gynecology, The University of Chicago, and the Chicago Lying-in Hospital.

Strains of gonococci after isolation and establishment on culture media, exhibit considerable difference in viability. Ordinarily it is necessary to make transfers of recently isolated strains at least twice a week and of older strains once a week. The maintenance therefore, of even a small stock collection of *N. gonorrhoeae* strains involves a considerable amount of time, labor, and expense.

Lumière and Chevrotier,[1] Szilvasi,[2] Cohn,[3] Parish,[4] and others have reported the preservation of an occasional strain or two of gonococci for from several weeks to 10 or 12 months. In most cases the strains were reported to have been grown on ordinary or blood agar, which meant necessarily that they were hardy strains. No one seems to have made a systematic controlled study to determine whether it was possible to preserve all strains of gonococci and sensitive ones in particular.

It had been our experience that once growth had been established

---

[6] Derrien, Eugene, and Turchini, Jean, *Comp. Rend. Biol.*, 1924, **91**, 637.

* This work has been done under a grant from the Albert B. Kuppenheimer Foundation.

[1] Lumière, A., and Chevrotier, J., *Compt. rend. Acad. Sci. Paris*, 1914, **158**, 1820.

[2] Szilvasi, J., *Dermat. Wehnschr.*, 1932, **94**, 245.

[3] Cohn, A., *Z. f. Hyg. u. Infekt.*, 1928, **108**, 395.

[4] Parish, H. J., *J. Path. and Bact.*, 1932, **35**, 143.

in Huntoon's[5] semi-solid stab cultures, practically all gonococci would remain viable for a month or more, but it required 3 to 15 transfers before some of the strains would grow in this medium. Repeated transfers are known to alter cultural characteristics and so a method of preservation which could be used immediately after isolation was sought.

Lumière and Chevrotier[1] reported that they had maintained gonococci viable for several months if the cultures were kept either *in vacuo* or sealed with paraffin oil or vaseline. Morton and Pulaski[6] had considerable success with mineral oil seals in the maintenance of a large collection of stock cultures of many bacterial species. They reported but one strain of *N. gonorrhoeae.* It had been grown on blood agar and was still alive at the end of 13 months. This method has the advantage that it not only preserves the cultures but keeps them readily available at all times for multiple transfer.

In order to determine the efficacy of this method of preservation, 30 strains of gonococci of varying age and sensitivity, isolated from several sources were studied over a period of more than 2 years. The age of the strains varied from 1 week to 8 years. Two showed slight growth on nutrient agar, and 17 grew fairly well on blood agar. Five of the strains were from male patients, 23 from female (18 from the cervix, 3 from the urethra, 2 from Bartholin's gland) 1 was isolated from a baby's eye and 1 from fluid aspirated from a thumb joint.

The results obtained were compared with cultures preserved in stoppered tubes and in Huntoon's stab cultures. In Huntoon's stabs the strains could be kept alive without transfer from 4 to 33 weeks.

Half the strains were lyophilized according to the method of Flosdorf and Mudd.[7] The lyophilized strains were prepared from 18 hour growths on McLeod's chocolate agar and suspended in sterile sheep serum. Only 4 of the 15 strains were alive at the end of 7 months. It is possible that better results might have been obtained by this method had the organisms been suspended in some material other than sheep serum.

The method of mineral oil seals was that described by Morton and Pulaski. Short slants of actively growing, young (usually not older than 18 hours) cultures were completely covered with sterile mineral oil. Heavy mineral oil (Parke, Davis and Co.) sterilized in a hot air oven at 150-170° for one hour was used. The tubes were stored in an incubator at 35.5° C. Several media were used; (1) McLeod's chocolate agar,[8] (2) McLeod's base with heated serum rather than whole blood, (3) Huntoon's semi-solid agar, (4) Difco's proteose peptone No. 3—hemoglobin agar and (5) Difco's dextrose starch agar. All the media gave practically the same results, except that those which remained viable longest were in Huntoon's agar. That, however, may have been because they were hardier and had been altered sufficiently before growth could be sustained in that medium. Monthly transfers were made from the original mineral oil covered slants for from 14-18 months. After 12 months all strains were transferred and then not transferred again for another 12 months. All 30 strains have survived 2 transfers made at 12-month intervals.

---

[5] Torrey, J. C., and Buckell, G. T., *J. Inf. Dis.*, 1922, **31**, 125.

[6] Morton, H. E., and Pulaski, E. J., *J. Bact.*, 1938, **35**, 163.

[7] Flosdorf, E. W., and Mudd, S., *J. Immunol.*, 1935, **29**, 389.

Better results were obtained when the tubes were stoppered with both cotton plugs and rubber stoppers than if cotton plugs alone were used.

Since this study was begun over 400 other strains have been isolated and placed under oil the second generation after isolation. Many of them have been transferred after 3- and 6-month intervals and found to be alive.

Conclusions. Strains of *N. gonorrhoeae* preserved by sterile mineral oil seals have been shown to be viable after transfer at 6 and 12 month intervals.

11690

Cell Opacity Method for Determination of Cell Volume on a Single Drop of Rat Blood.*

A. T. Shohl, K. D. Blackfan and L. K. Diamond.

From the Department of Pediatrics, Harvard Medical School, and the Infants' and the Children's Hospitals, Boston.

The rat is admirably adapted to the study of certain problems in the physiology and pathology of blood. It has been used extensively in experimental nutritional anemia. For such studies it is desirable to know not only the hemoglobin content and red cell count of the blood but also the volume of the packed red cells. Sufficient blood for the first two determinations is easily obtainable by incision of the tail vein, but not for the third determination, with methods at present available. The red cell volume can be determined by the widely used hematocrit method. This requires 1 cc of blood and it is necessary to sacrifice the animals by bleeding. This procedure, however, precludes a series of observations on the same animal. A method for determining red cell volume on a small amount of blood is therefore especially desirable.

---

[8] McLeod, J. W., Coates, J. C., Happold, F. C., Priestley, D. P., and Wheatley, B., *J. Path. and Bact.*, 1934, **39**, 221.

* This study was supported in part by a grant from the Rockefeller Foundation.

A method is here proposed for the determination of red cell volume on a single drop of rat blood, similar to that of human blood.[1] Hemoglobin content may be determined on the same sample.[2] Erythrocyte count may be made at the same time. Thus all the information desired may be secured without killing the animal or withdrawing enough blood to cause depletion which would alter its subsequent condition.

The method for cell volume, which we have called the cell opacity method, depends upon the measurement of the transmission of light through a suspension of blood cells in citrate solution. The Evelyn photoelectric colorimeter was used with a filter transmitting light at or near 660 mμ. The light transmitted impinges upon a photoelectric cell and the amount of current developed is measured on a galvanometer. Other types of photoelectric colorimeter may be employed. The impedance to light is directly related to the cell volume. The galvanometer reading can be converted to cell volume from a plot showing the relation of the two values (Fig. 1). Sub-

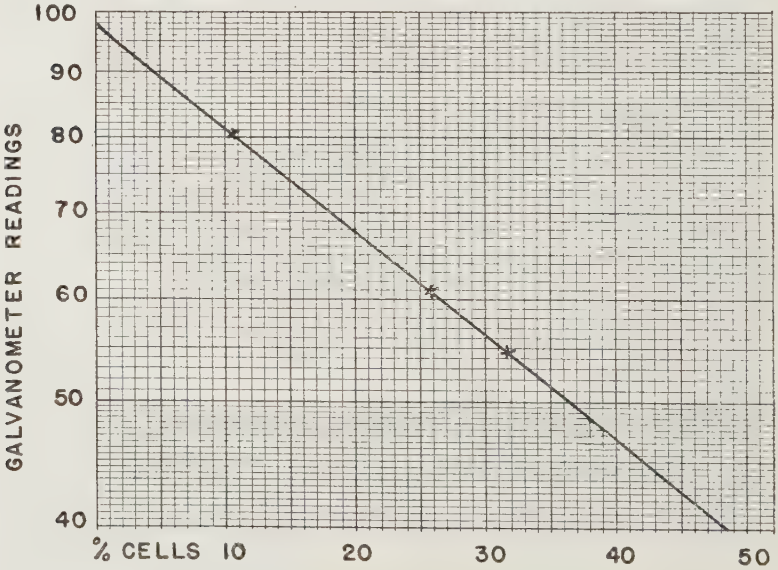


FIG. 1.
See text for description.

---

[1] Shohl, A. T., *J. Lab. and Clin. Med.*, 1940, **25**, 1325.

[2] Evelyn, K. A., *J. Biol. Chem.*, 1936, **115**, 63.

sequently the same sample of blood may be hemolyzed by addition of saponin and ammonia, and the hemoglobin may be quantitated by measuring the transmission of light with the substitution of a filter which transmits light in the region of 540 mμ.[2]

The optical properties of rat blood differ slightly from those of human blood. Normal rat blood has approximately 8 million red cells per cm compared to 5 million in human blood, but has nearly the same red cell volume. The individual cells are therefore smaller. The studies of Drabkin and Singer[3] showed that, although the size of the individual particles is a factor in the scattering of light, the total volume and not the number of cells is the main determinant of the transmission of light through a cell suspension.

Experimental. Inasmuch as the correlation between the transmission of light and the volume of the packed cells is empirical, it was necessary to make comparative determinations on aliquots of the same blood by an alternative method, and thus to establish a standard for interpretation of galvanometer readings. Once this standard is established the cell opacity method may be used independently of another method.

In order to establish this correlation, the blood of each of 44 rats was analyzed by both methods. The blood was obtained from albino rats of Wistar Institute stock, bred in our own laboratory. The animals were anesthetized with ether, bled from the femoral vessels and the blood collected in graduated tubes containing sufficient dry oxalate (60% ammonium oxalate and 40% potassium oxalate) to make a final concentration of 0.2%. Aliquots were taken for the two methods.

The standard of reference used was the hematocrit method described by Wintrobe.[4] By this technic oxalated blood is centrifuged in a tube with 100 marked divisions at 3000 R.P.M. for 30 minutes and the red cell volume read directly in percentage.

The technic for the cell opacity method is as follows: In a hemoglobin pipette calibrated "to contain", 20 cm of the oxalated blood are measured. This is delivered into and the pipette washed with 8 cc of citrate solution contained in a test tube selected to fit the colorimeter.† The diluent consists of 3% anhydrous sodium citrate (4.15% Na citrate. 5½ H_2O). One cc of formalin (36-38% formaldehyde gas) per liter is added before making the citrate solution up to volume. The tube is placed in the photoelectric colorimeter after the galvanometer has been adjusted to read 100 with a "blank" tube containing the diluent only. The reading is made after 15-30 seconds. The galvanometer reading is then translated into cell volume from the curve given in Fig. 1, which was established from the experimental data. The ordinates represent the logarithm of the galvanometer readings, and the abscissae the volume of the packed red cells by the hematocrit method. The intercept of the galvanometer reading and the cell volume was plotted for each of the 44 determinations. Fifteen determinations were made with 10 cmm of blood and 29 with 20 cm. The two crosses represent the arithmetical mean calculated for each of the 2 groups. The point of 0 cells was taken as 98, the galvanometer reading for plasma alone, and a line drawn from this point through the crosses. This was seen to be a representative line.

---

[3] Drabkin, D., and Singer, R. B., *J. Biol. Chem.*, 1939, **129**, 739.

[4] Wintrobe, M. M., *J. Lab. and Clin. Med.*, 1929, **15**, 287.

† The new Evelyn colorimeters are equipped to use 6 or 10 cc of solution. The Klett-Summerson colorimeter uses tubes marked at 5 and 10 cc. The same concentration of blood is obtained if 15 cm are added to 6 cc or 25 cm to 10 cc of citrate solution. If other dilutions are used, the readings give corresponding fractional values. Complete directions for the use of the instrument are given in the manual supplied with the photoelectric colorimeter.

The calculation of variations from the line shows approximately equal distribution of + and — values. A plot of the variation between the values obtained by the two methods shows a normal distribution curve of variation. Two-thirds of the values fall within ±1.5 volumes per 100 cc of blood. This agreement is not quite as close as that previously reported for human blood.[1]

A series of 10 normal rats was studied in order to ascertain whether the values obtained by the cell opacity method using blood from the tail vein agreed with the values obtained when the animals were sacrificed by bleeding into tubes containing dry oxalate. In the latter procedure the blood is a mixture of arterial and venous blood. Determinations were made of the cell opacity, hemoglobin and red cell counts, in duplicate, on both cutaneous and mixed blood. Cell volume was determined also by the hematocrit method on mixed blood. All determinations were made on each rat.

The cutaneous blood gave values sometimes higher and sometimes lower than the mixed blood by either the cell opacity method or the hematocrit method. The variations were within the limits given in Fig. 1. Moreover, the variation in cell volume as determined by the hematocrit method and by the cell opacity method, either in the same sample or in comparison between the cutaneous and mixed blood, is less than the variation in the red cell count between the two types of blood or even between duplicate red counts on the same blood.

Summary. A method is presented for determination by the use of the photoelectric colorimeter of red cell volume of rat blood on a single drop of blood obtained from the tail vein. Hemoglobin content may be determined on the same sample.

Technical assistance of Dorrance Chase Deston is gratefully acknowledged.

11691

Specific Substances in the Urine of Leucemia Patients.

F. R. MILLER AND W. A. HAUSE.

From the Department of Medicine, Division of Hematology, Charlotte Drake Cardeza Foundation, Jefferson Medical College.

Miller, Wearn and Heinle[1] reported specific proliferations of cells in the organs of guinea pigs which had been given daily injections of extracts of urine from patients with chronic myeloid and lymphoid leucemias. In the course of further experiments with the same extracts attempts were made to further concentrate the substance. This was accomplished by reducing the pH of the final water extract to 1.5 or 2.0 with hydrochloric acid and shaking vigorously for 15 minutes with an equal amount of chloroform. The chloroform and water were separated and the chloroform removed by vacuum. The residue was resuspended in alkaline water at a pH of 7.4 to 7.6. Each cc of the extract was computed to equal from 1000 to 1500 cc of urine. These extracts were given to guinea pigs in step doses starting with ¼ cc and increasing every 5 days until 1½ or 2 cc was given daily. Animals on such regimes again presented specific pictures in 4 to 6 weeks.

Because we felt that neither of the preceding methods of extraction seemed to yield as much active material as was present another method has been evolved, and for this we have adapted a modification of McCullagh's method of extracting androgens from urine. The urines are hydrolyzed by boiling at a pH of 2.0. It has been found that such a pH in most instances could be obtained by the addition of 40 to 45 cc of concentrated hydrochloric acid to each 1000 cc of urine. After cooling 100 cc of chloroform is added to each 1000 cc of urine and this is stirred violently with an electric stirrer for 20 minutes. The chloroform is then separated and the process repeated 5 times. The separate parts of chloroform are added together and if an emulsion is present this is broken by the addition of a few cc of 10% sodium taurocholate solution. After filtering the chloroform is then distilled off leaving a brown black greasy substance. This substance is then emulsified in water by the addition of a small amount of 5% sodium hydroxide. The emulsion is brought to a pH of 7.4 to 7.6 and after it is suspended in this manner it is boiled for 20 minutes and is ready for use. Each cc of the emulsion is equal to 60 cc of urine. This extract is obtained from a smaller amount of urine than the final solution made by the second method, however each cc contains more solid material.

---

[1] Miller, F. R., Wearn, J. T., and Heinle, R. W., PROC. SOC. EXP. BIOL. AND MED., 1939, **41**, 478.

Such extracts of urine from patients with chronic myeloid leucemia were given in step doses to 4 guinea pigs. One 1-eighth cc was used as the initial daily dose and every 5 days the dose was increased until a dose of 1½ or 2 cc daily had been reached. Within 4 to 6 weeks the animals had lost weight, were in ill health so that they were either sacrificed or died. Each of these animals presented myeloid proliferations in liver, spleen, adrenal, lung and kidney similar to those mentioned in the earlier report. Two of these animals had spleens which weighed 3.5 g.

Similar extracts of urine from one patient with acute lymphoblastic leucemia and the extracts of urine from one patient with chronic lymphoid leucemia were given to 2 pairs of guinea pigs. One of each pair of these guinea pigs died in 2 weeks on doses similar to those given the extract from urine of patients with chronic myeloid leucemia. In these there was marked lymphadenopathy as well as lymphocytic proliferation in the liver, spleen and adrenal. The animal which received the material from the acute leucemia exhibited amaturation of the bone marrow. The other 2 animals died in 5 weeks with findings similar to the first 2 but these were not so marked.

Extracts of urine of 2 normal individuals gave no specific cellular response.

Conclusions. By a modification of McCullagh's[2] method for extracting androgen from urine we have been able to improve the method of extracting specific substances from the urine of leucemia patients. These substances give specific cellular reactions in guinea pigs. We are now testing saponifiable and non-saponifiable fractions of the chloroform residue which have been prepared by Hause.

---

[2] McCullagh, D. R., and McLin, T. R., *Endocrinology*, 1938, **22**, 120.

11692

Circling in Guinea Pigs with Lesions in the Brain Stem.

A. R. Buchanan.

From the Department of Anatomy, University of Colorado, School of Medicine, Denver.

Lurie[1] recently described circling or "waltzing" in guinea pigs; the circling was associated with deafness and the combined symptoms comprised an inherited characteristic of the recessive type. He concluded, since the peripheral vestibular mechanism was normal, that the circling was due to some disarrangement of a central nervous mechanism, probably in the region of the red nucleus.

Lurie and Dempsey[2] observed circling in guinea pigs with lesions in the interpeduncular region, immediately caudal to the mammillary bodies; a lesion on the left side of the midline induced circling to the right. They believed the circling in these animals to be due to a break in the normal reflex arc between the vestibular apparatus, the third nerve nucleus, and the postural centers.

The experimental observations and postulations of Lurie and Dempsey have made more significant some heretofore unreported observations on a group of my experimental guinea pigs. Circling has been one of the symptoms resulting from lesions in the medulla and pons in 13 animals; since the peripheral vestibular mechanism was intact and there was a break in the vestibulo-ocular reflex arc in each of these animals, a report of the phenomena seems warranted.

All the lesions were produced with Horsley-Clarke stereotaxic instruments and all were unilateral. Four were placed in the right side of the brain stem and the remaining 9 were on the left of the midline.

Tabulation of the observations (Table I) has revealed the interesting fact that circling was toward the side of the lesion in 6 animals and toward the opposite side in 7. Further analysis (see table) has shown a definite correlation, even in this small group of animals, between the location of the lesion and the direction of circling. Of those which circled toward the side of the lesion, 3 had involvement of Deiters' nucleus and 2 had lesions in the descending vestibular nucleus; the sixth in this group had partial destruction of the medial vestibular nucleus which was complicated by a second lesion in the medial nucleus of the cerebellum.

---

[1] Lurie, M. H., *The Laryngoscope*, 1939, **49**, 558.

[2] Lurie, M. H., and Dempsey, E. W., *The Laryngoscope*, 1939, **49**, 565.

TABLE I.
Circling in Guinea Pigs Following Lesions in Brain Stem.

Animal No.	Location of lesion	Side of lesion	Direction of circling	P.O. day first observed	Duration in days
1	D.Nu.; Desc.Nu.	Right	Right	5	1
2	D.Nu.; M.Nu.; J.R.B.	Left	Left	2	1
3	D.Nu.; M.Nu.	,,	,,	2	2
4	Desc.Nu.	,,	,,	2	4
5	Desc.Nu.	,,	,,	1	1
6	M.C.Nu.; M.Nu.	Right	Right	3	1
7	M.Nu.	Left	,,	2	5
8	M.Nu.; M.R.F.	,,	,,	2	1
9	M.Nu.; M.R.F.	,,	,,	1	5
10	M.R.F.	,,	,,	2	1
11	M.R.F.	,,	,,	2	1
12	M.L.F.	Right	Left	1	5
13	M.Nu.; N.I.; D.Nu.; Desc.Nu.	,,	,,	2	3

Abbreviations: Desc.Nu.—Descending vestibular nucleus; D.Nu.—Deiters' nucleus; J.R.B.—Juxtarestiform body; M.C.Nu.—Medial cerebellar nucleus; M.L.F.—Medial longitudinal fasciculus; M.Nu.—Medial vestibular nucleus; M.R.F.—Medial reticular formation; N.I.—Nucleus interpositum (cerebellum); P.O.—Post-operative.

Circling toward the side opposite that of the lesion was brought about in one animal by a lesion confined to the medial vestibular nucleus, in 2 others by lesions in the same nucleus with concomitant involvement of the medial reticular formation, and in 2 by lesions entirely within the medial reticular formation. Another proved to have a lesion involving the medial longitudinal fasciculus at the level of the superior vestibular nucleus and the last animal in this group had the greatest amount of destruction in the medial vestibular nucleus with concomitant injury to the nucleus interpositum in the cerebellum, Deiters' nucleus, and the descending nucleus.

Lesions in Deiters' nucleus or in the medial nucleus most certainly interrupt the connections between the vestibular mechanism and the third nerve nucleus, thus supporting the theory presented by Lurie and Dempsey, even though they are at a more caudal level than the lesions produced by these workers. The lesion in the medial longitudinal fasciculus also interrupted the vestibulo-ocular reflex arc. On the basis of the reports by Lorente de No'[3] and others describing connections from the vestibular nuclei to the reticular cells and thence to the motor ocular nuclei, the assumption can be safely made that lesions within the reticular formation also disturb this same reflex arc. Furthermore, it is quite probable that the connections from the vestibular nuclei to the "postural centers" mentioned by Lurie and Dempsey are completed through the medium of the reticular cells.

---

[3] Lorente de No', R., *Ergebn. Physiol.*, 1931, **32**, 73; *Arch. Neur. and Psychiat.*, 1933, **30**, 245.

Since I have been unable to demonstrate, in Marchi preparations, any ascending fibers in the medial longitudinal fasciculi which have their origin in the descending vestibular nuclei, either in cats[4] or in guinea pigs (unpublished observations), one must assume here, also, a connection with postural centers and motor ocular nuclei through the reticular cells. The more or less paradoxic direction of circling in the 2 animals (6 and 13), having lesions in the cerebellum and vestibular nuclei is probably explained by the complex interrelationship between cerebellum and vestibular nuclei.

Other postural defects, spontaneous nystagmus, etc., were also observed in this group of animals, similar to those reported elsewhere.[5]

The relatively short duration of the circling phenomena in most of the animals indicates an efficient compensatory mechanism. The late appearance of circling in animals 1 and 3 was due to their inability to assume the upright position previously. It is of interest that hypotonia, when it was evident, was always on the side of the lesion, even in those animals which circled to the opposite side.

Summary. Circling to one or the other side has been observed in 13 animals with lesions in the medulla and pons. Lesions in Deiters' and in the descending vestibular nucleus produced circling toward the side of the lesion, while those in the medial vestibular nucleus, in the medial reticular formation, and in the medial longitudinal fasciculus resulted in circling toward the opposite side. Since the connections between the vestibular mechanism and the motor ocular nuclei and possibly, those between the former and some postural centers were interrupted, these observations are reported as confirmatory of the hypothesis put forward recently by Lurie and Dempsey to explain the circling observed in their experimental guinea pigs.

---

[4] Buchanan, A. R., *J. Comp. Neur.*, 1937, **67**, 183.

[5] Buchanan, A. R., *Monatschr. f. Psychiat. u. Neur.*, in press; *The Laryngoscope*, 1937, **47**, 874; *The Laryngoscope*, in press.

11693

Influence of Increased Environmental Temperature on Blood Sugar of Dogs Following Administration of Carbohydrate.

M. A. Rafferty and P. L. MacLachlan. (Introduced by E. J. VanLiere.)

From the Department of Biochemistry, School of Medicine, West Virginia University, Morgantown.

Studies concerning the effect of increased environmental temperature on the blood sugar content of different animals have led to widely varying conclusions. Schear[1] found in albino rats that the concentration of the blood sugar and the environmental temperature stand in an inverse relation to each other. As the temperature rises the blood sugar level lowers, and vice versa. On the other hand, Weyl[2] found that neither long nor short exposure to high temperature caused either hypoglycemia or hyperglycemia in dogs, and concluded that the statement that the blood sugar level is lower at high temperatures than at low temperatures is untenable. Flinn and Scott[3] likewise found no change in the blood sugar content of dogs exposed to increased temperatures.

The present work was undertaken to determine the effect of exposure to high environmental temperature on the blood sugar content of dogs following the administration of varying amounts of carbohydrate in order to gain a better knowledge of the specific effects of high temperature on digestion and absorption.

Four adult female mongrel dogs were maintained on a diet of table scraps supplemented with Purina dog chow for at least 2 weeks prior to the experiment. Following a 24-hour fast the experimental dog was placed in a respiratory chamber (connected to a pump which provided adequate ventilation and removal of CO_2) at a temperature of 100°-104° and an average humidity of 77.5% for 1½ hours before the fasting blood sample was drawn. An aqueous solution of glucose or suspension of starch was then given by stomach tube in varying amounts per kg body weight (Table I). The dog was returned to the respiratory chamber and blood samples for glucose determination were drawn from a leg vein at hourly intervals for 3 to 4 hours. The control dog was treated similarly at a room temperature of 68°-77° and an average barometric pressure

---

[1] Schear, E. W. E., *Am. J. Physiol.*, 1932, **99**, 555.

[2] Weyl, P., *Biochem. Z.*, 1929, **206**, 485.

[3] Flinn, F. B., and Scott, E. L., *Am. J. Physiol.*, 1923, **66**, 191.

TABLE I.

Effect of Environmental Temperature on the Blood Sugar of Dogs Following the Administration of Glucose or Starch.

Dog No.	Body wt, kg	Carbohydrate		Blood sugar					Environmental Temperature °F
		Kind	g/kg	Fasting mg%	1 hr mg%	2 hr mg%	3 hr mg%	4 hr mg%	
				Control.					
1	8.0	Glucose	1.75	86	92	66	72	—	70
1	8.0	,,	1.75	74	124	71	55	—	68
1	8.0	,,	1.75	76	119	70	—	—	73
1	8.0	Starch	1.75	70	85	67	65	—	69
2	7.0	,,	6.00	73	104	106	86	89	75
2	7.0	,,	6.00	—	106	105	111	89	77
3	4.7	,,	6.00	77	95	95	90	94	77
4	5.0	,,	16.00	75	94	105	90	93	74
Avg				76	102	86	81	91	73
				Experimental.					
4	5.0	Glucose	1.75	81	89	67	70	—	100
4	5.0	,,	1.75	77	87	79	78	—	102
4	5.0	,,	1.75	70	125	72	—	—	101
4	5.0	Starch	1.75	81	79	80	74	—	100
1	8.0	,,	6.00	83	85	120	107	97	102
1	8.0	,,	6.00	68	87	101	97	77	101
4	5.0	,,	6.00	74	103	103	108	101	104
3	4.7	,,	16.00	75	107	99	118	97	103
Avg				76	95	90	93	93	102

of 740 mm. Blood sugar determinations were made by standard procedures, Folin and Wu.[4]

The experimental dogs were subjected to an environmental temperature of approximately 29 degrees higher than the controls. The average rectal temperature of the control animals was 101.4 degrees. As a result of the higher environmental temperature the rectal temperature of the experimental animals increased from approximately 101 to 105 degrees. However, the results, Table I, show that there were no significant differences between the blood sugar values of the control and the experimental animals following the administration of either glucose or starch. In both control and experimental animals the blood sugar level was more sustained when larger amounts of carbohydrate were given.

The fasting blood sugar level of the experimental dogs, which was determined after 1½ hours of heat treatment, showed no difference from the normal fasting level. This is in agreement with the observations of Flinn and Scott[3] and Weyl,[2] that increased temperature is without effect on the blood sugar content.

Summary. These findings indicate that exposure to high environmental temperature does not influence the digestion and absorption of carbohydrate in the dog, as far as the blood sugar content is concerned. Moreover, no relation is apparent between the blood sugar level and increased environmental temperature.

11694

Effect of Hepatic Injury on Vitamin C Excretion in Fasting Dogs.*

A. T. MILHORAT, W. E. BARTELS AND V. TOSCANI.

From the Russell Sage Institute of Pathology in Affiliation with the New York Hospital and the Departments of Medicine and Psychiatry, Cornell University Medical College, New York City.

Fasting dogs normally excrete a urinary substance that appears to be identical with Vitamin C. This substance reduces 2:6 dichlorphenol indophenol and gives the color reaction used in the estimation of the vitamin. Moreover, it was established in these investigations that the reducing substance further resembles vitamin

---

[4] Folin, O., and Wu, H., *J. Biol. Chem.*, 1920, **41**, 367.

* Aided by a grant from the National Foundation for Infantile Paralysis, Inc.

C in that it is not precipitated by mercuric acetate and is destroyed by incubation with the pressed juice of cauliflower. Dogs given a normal laboratory diet excreted increased amounts of substances that reduced the indophenol, but most of these could be removed from the urine by precipitation with mercuric acetate. After the dogs had been put on a fast, the excretion of reducing substance decreased over a period of about 4 days and thereafter the elimination remained constant throughout the period of fasting which usually was from 21 to 30 days.

The biological identity of the reducing substance excreted by the fasting dog has not been established, but Musulin, Tully, Longenecker and King[1] have shown that the reducing substance excreted in the urine by rats has antiscorbutic properties.

Experimental. Female dogs were fasted for a preliminary control period of about 10 days and for the duration of the experiment but were allowed free access to drinking water. The animals were kept in metabolism cages permitting quantitative collection of all urine in dark colored bottles containing acetic acid. At the end of each 24-hour period the dogs were catheterized.

Hepatic injury was induced by 3 different methods. One method was the induction of deep chloroform anesthesia by inhalation for one hour, and another was the administration of carbon tetrachloride by stomach tube.

In the third type experiment, various substances were administered in an investigation of their effects on urinary metabolism. Shortly following the administration of a hexane extract of oat oil the dog developed deep jaundice and evidence of severe hepatic injury. This animal had been studied repeatedly over a period of 4 years. During all the periods of study up to the onset of the hepatic damage the urinary metabolism had remained constant and all evidence of liver involvement had been lacking. While the causative agent is still being investigated the results were so striking that their inclusion in this report is justified. Histological study of the liver tissue showed an advanced atrophy of hepatic cells and an early increase in connective tissue.

Observations. The data observed in representative experiments on the effects of chloroform and carbon tetrachloride are shown in Table I. Chloroform inhalation induced definite increase in the excretion of both urinary nitrogen and vitamin C.

The effect of carbon tetrachloride was not uniform. In some experiments the excretion of the vitamin was increased, but in others

---

[1] Musulin, R. R., Tully, R. H., 3d, Longenecker, H. E., and King, G. G., *J. Biol. Chem.*, 1939, **129**, 437.

TABLE I.
Effect of Chloroform Anesthesia and of Carbon Tetrachloride.

Day of fast	Daily urinary excretion		Gmelin test	Remarks
	Total N, g	Vitamin C, mg		
33	2.52	26	0	
34	2.38	26	0	
35	3.98	56	+	Chloroform anesthesia one hr
36	3.27	28	+	
37	2.75	18	±	
8	1.99	36	0	
9	1.99	32	0	
10	2.26	26	0	14 cc C Cl_4 *per os.*
11	2.87	12	+	
12	2.51	8	+	
16	1.78	4	0	
17	1.68	4	0	
18	2.02	13	+	10 cc C Cl_4 *per os.*
19	2.75	11	+	
20	2.27	9	±	

the excretion was unaffected for the first day or two and then was decreased for several days.

Table II shows the data observed in severe hepatic injury. For comparison, observations made during a normal period are included. The data show that during the period of liver damage the vitamin C and creatine outputs were unusually large, the excretion of urea and creatinine was decreased, the urinary ammonia was abnormally high despite absence of infection, and an impaired ability to deaminize amino acids was demonstrated by the high output of amino nitrogen after administration of protein hydrolysate.

TABLE II.
Effect of Advanced Hepatic Injury on Daily Urinary Output.

Day of fast	Total N, g	Urea N, g	NH_4 N, mg	Amino N, mg	Creatinin, mg	Creatin, mg	Vit. C, mg
Control Period, December, 1938.							
15	1.65	1.04	78	32	101	55	42
16*	2.10	1.26	73	26	135	45	30
17	1.54	1.12	84	18	109	29	32
Period of Hepatic Injury, March, 1939.							
21	1.27	0.61	450	39	88	80	220
22†	1.99	0.77	450	366	89	94	176
23	1.40	0.57	390	36	98	86	270
24	1.25	0.56	420	40	81	100	260
25	1.25	0.59	460	67	83	101	280

*Given Protein Hydrolysate (0.67 g N) *per os.*
† ” ” ” (1.0 g N) ” ”

Gmelin Test was strongly positive on all urinary samples during period of hepatic injury, and always negative during control period.

The observations suggest that in the dog the liver plays an important part in the metabolism of vitamin C. The changes in the output of the vitamin seen after induction of hepatic injury are much larger than the normal daily variations or those observed after the administration of a wide variety of substances studied as possible precursors of vitamin C, such as proteins, amino acids, carbohydrates and various vegetable and animal oils. It is likely the increase in vitamin C output following chloroform anesthesia was in part due to asphyxia and subsequent depletion of tissue glycogen.

The function of the liver that is concerned with vitamin C metabolism is not known, but the investigations on shivering and iodoacetate reported in an accompanying paper[2] suggest that the glycogenetic function is the one involved.

11695 P

Effect of Shivering, Iodoacetate, and Epinephrine on Vitamin C and Creatine Excretion in Fasting Dogs.*

A. T. MILHORAT, J. D. HARDY, W. E. BARTELS AND V. TOSCANI.

From the Russell Sage Institute of Pathology in Affiliation with the New York Hospital and Departments of Medicine and Psychiatry, Cornell University Medical College, New York City.

In a previous report[1] the excretion of vitamin C by the fasting dog was discussed, and it was shown that hepatic injury can increase the urinary output of the vitamin. In the following experiments, the effect of severe shivering, epinephrine, and iodoacetate on the excretion of various urinary substances was investigated.

Experimental. The technic was similar to that described previously.[1]

Shivering was induced by anesthetizing the animals with intravenous nembutal and then placing them in a refrigerator at 12°C. In most instances the body temperature fell to around 31°C. Although additional nembutal kept the animals in an unconscious state, severe and almost constant shivering occurred.

---

[2] Milhorat, A. T., Hardy, J. D., Bartels, W. E., and Toscani, V., PROC. SOC. EXP. BIOL. AND MED., 1940, **45**, 397.

* Aided by a grant from the National Foundation for Infantile Paralysis, Inc.

[1] Milhorat, A. T., Bartels, W., and Toscani, V., PROC. SOC. EXP. BIOL. AND MED., 1940, **45**, 394.

Observations. Data in typical experiments are shown in Table I. Shivering produced considerable increase in the excretion of vitamin C and creatine, an increase in urinary nitrogen and urea and a decrease in creatinine.[†] Following periods of shivering lasting from 24 to 72 hours, the animals developed rigid paralysis of the extremities which persisted until food was given. One dog that was not fed had severe stiffness and paralysis of the extremities for 7 days when it died apparently with weakness of the muscles of respiration. In all animals muscular paralysis was accompanied by large output of urinary creatine and vitamin C.

The effect of nembutal in animals kept at warm room temperatures was determined. Complete anesthesia associated with slight shivering induced definite increase in urinary nitrogen but only a slight increase in vitamin C excretion and was without effect on the creatine.

Iodoacetate. Because of the similarity between the rigid paralysis induced by severe shivering and that seen in animals poisoned with iodoacetate, the effect of iodoacetate was studied. The data presented in Table I show that the administration of iodoacetate induced definite creatinuria but did not change the output of vitamin C.

TABLE I.
Effect of Shivering and of Iodoacetate on Daily Urinary Output.

Day of fast	Total N, g	Urea N, g	Creatinin, mg	Creatin, mg	P, mg	Vitamin C, mg
15	1.66	1.22	165	20	138	48
16	1.46	1.17	188	5	105	48
17*	Urine lost					
18*	1.44	1.14	96	56	134	124
19†	1.89	1.41	127	155	63	148
20†	2.38	1.91	152	165	90	120
21†	1.80	1.39	171	80	49	110
22†	1.76	1.36	156	43	74	60
17	2.52		310	36	132	60
20	2.17		357	5	139	68
21‡	3.05		375	28	222	64
22	3.42		327	160	94	62
23	2.55		307	84	96	40
24	1.76		284	0	88	40

*Severe shivering for 24 hrs.
†Rigid paralysis of extremities.
‡Iodoacetic acid 100 mg intrav.
Blood sugar level (mg per 100 cc):

Before shivering	55
1st day of shivering	108
2nd day of shivering	58

[†] The variations in the different experiments were: Vitamin C, from 80 to 200 mg; creatine, 70 to 140 mg; total nitrogen, 0.8 to 1.8 g; urea N, 0.7 to 1.7 g; creatinine, 70 to 110 mg.

Epinephrine. The administration of epinephrine increased the urinary excretion of nitrogen, vitamin C, and creatine.

Discussion. It is of interest that both severe shivering and epinephrine increased the urinary output of vitamin C and creatine whereas iodoacetate given in amounts large enough to induce definite creatinuria did not change the excretion of vitamin C. Both shivering and epinephrine are known to remove glycogen from the muscles (Külz,[2] Junkersdorf[3]), whereas iodoacetate interferes with the glycogen-lactic acid cycle at the hexose monophosphate stage but appears not to affect the glycogen store itself. In these experiments shivering and epinephrine increased the blood sugar and induced glycosuria. The large output of creatine probably was the result of impaired resynthesis of creatin-phosphoric acid secondary to the defect in the glycogen-lactic acid cycle. In some experiments the urinary excretion of phosphorus was increased during the first day. On subsequent days, despite appreciable creatinuria, the phosphorus output was not increased. It is probable that some of the phosphoric acid was retained as hexosephosphate esters (Cori and Cori[4]).

The data suggest that the metabolism of vitamin C is related significantly to that of glycogen. Moreover, the stages in glycogen metabolism that are involved in this relationship appear not to be those in the lactic acid cycle that are at or beyond the hexosemonophosphate stage, since factors that remove muscle glycogen increase the urinary vitamin whereas iodoacetate is without effect. This formulation would explain the findings of other investigators on the effect of ether anesthesia (asphyxia) (Bowman and Muntwyler,[5] Zilva,[6]) and insulin (Ralli and Sherry[7]) on vitamin C excretion.

---

[2] Külz, E., C. Ludwig's *Festschrift*, Marburg, 1891, p. 119.

[3] Junkersdorf, P., and Török, P., *Pflugers Arch. gesam. Physiol.*, 1926, **211**, 414.

[4] Cori, G. T., and Cori, C. F., *J. Biol. Chem.*, 1936, **116**, 119.

[5] Bowman, D. E., and Muntwyler, E., PROC. SOC. EXP. BIOL. AND MED., 1935, **33**, 437.

[6] Zilva, S. S., *Biochem. J.*, 1935, **29**, 2366.

[7] Ralli, E. P., and Sherry, S., PROC. SOC. EXP. BIOL. AND MED., 1940, **43**, 669.

11696 P

Typing of Typhoid Bacilli in Nine Western States by Bacteriophage Method of Craigie and Yen.

Alfred S. Lazarus. (Introduced by Ivan C. Hall.)

From the Department of Bacteriology and Public Health, University of Colorado School of Medicine and Hospitals, Denver.

The method of typing various strains of *Bacterium typhosum,* as reported by Craigie and Yen,[1] has received neither the recognition nor the widespread application that its useful possibilities seem to justify. The survey herein reported was undertaken in an effort to bring the advantages of this method to the attention of those most likely to benefit by its use.

The typing method is based on certain peculiarities of a strain of bacteriophage which is specific for the Vi form of *B. typhosum.* In addition this bacteriophage exhibits a selective affinity for the strain on which it is propagated and for epidemiologically related strains. By selective propagation of this bacteriophage on various strains of *B. typhosum,* Craigie and Yen[1] have shown that it is possible to divide freshly isolated cultures into a limited number of readily distinguished types, which have been designated by the letter system A to J inclusive, with the exception of type I. Later reports by Craigie[2, 3] and by Yen[4] have added subtypes of B, D, E, and F and additional studies have brought to light types L and M, which are rarely encountered.[5] Among the typed cultures herein reported, 97.1% fell into the 6 main groups, A, B, C, D, E, and F.

TABLE I.
Distribution of Cultures by States.

	A	B_1	B_2	C	D_1	E_1	F_1	J	L	M	Imp. V	W	Totals
California	15	1	4	7	—	22	40	2	1	1	13	2	108
Colorado	—	3	1	2	—	4	1	—	—	—	1	—	12
Idaho	—	—	—	1	—	—	1	—	—	—	—	—	2
Montana	—	1	4	—	—	2	—	—	—	—	—	4	11
Nevada	—	—	—	1	—	—	—	—	—	—	—	—	1
New Mexico	5	6	24	2	7	32	16	—	—	—	12	1	105
Oregon	24	1	4	7	5	14	10	4	—	—	6	1	76
Utah	1	—	2	10	1	—	—	—	—	—	—	2	16
Washington	7	1	1	—	2	7	—	1	—	—	—	9	28
Totals	52	13	40	30	15	81	68	7	1	1	32	19	359

---

[1] Craigie, J., and Yen, C. H., *Canad. Public Health J.*, 1938, **29**, 448.

[2] Craigie, J., *Canad. Pub. Health J.*, 1939, **30**, 37.

[3] Craigie, J., *Canad. Pub. Health J.*, 1940, **31**, 18.

[4] Yen, C. H., Proc. Soc. Exp. Biol. and Med., 1939, **41**, 162.

[5] Craigie, J., personal communication.

Cultures for this study were obtained through the coöperation of the directors and technical staffs of the 9 state laboratories concerned, and were typed by the method described in detail in the original report by Craigie and Yen.[1] Table I shows the distribution of the various types by states.

Table II is included to show the distribution of individual patients by states, since in many cases multiple cultures were received from single individuals. It will be noted that types B_3, B_4, D_2, D_3, E_2, F_2, G and H have not as yet been encountered in the area being studied.

TABLE II.
Distribution of Individuals by States.

										Imp.			%
	A	B_1	B_2	C	D_1	E_1	F_1	J	L	M	V	W	Totals	typed
California	12	1	4	6	—	16	37	2	1	1	7	2	89	90
Colorado	—	3	1	1	—	4	1	—	—	—	1	—	11	91
Idaho	—	—	—	1	—	—	1	—	—	—	—	—	2	100
Montana	—	1	3	—	—	1	—	—	—	—	—	2	7	71
Nevada	—	—	—	1	—	—	—	—	—	—	—	—	1	100
New Mexico	5	5	17	2	5	23	11	—	—	—	3	1	72	94
Oregon	16	1	3	6	3	9	8	3	—	—	5	1	55	89
Utah	1	—	1	10	1	—	—	—	—	—	—	2	15	87
Washington	7	1	1	—	2	7	—	1	—	—	—	9	28	68
Totals	41	12	30	27	11	60	58	6	1	1	16	17	280	88.2

Table III demonstrates the number of cultures isolated from each of the 280 individuals concerned.

TABLE III.
Number of Isolations per Individual.

No. of times isolated per individual											Imp.		
	A	B_1	B_2	C	D_1	E_1	F_1	J	L	M	V	W	Totals
1	34	11	23	24	8	48	51	5	1	1	11	15	232
2	6	1	6	3	2	6	4	1	—	—	1	2	32
3	—	—	—	—	1	4	3	—	—	—	3	—	11
4	—	—	—	—	—	1	—	—	—	—	—	—	1
5	—	—	1	—	—	1	—	—	—	—	—	—	2
6	1	—	—	—	—	—	—	—	—	—	—	—	1
10	—	—	—	—	—	—	—	—	—	—	1	—	1
No. of individuals	41	12	30	27	11	60	58	6	1	1	16	17	280
No. of cultures	52	13	40	30	15	81	68	7	1	1	32	19	359

It is to be noted that no evidence of unstability of type has as yet been encountered, either in the cultures of human origin herein tabulated or in supplementary animal experiments now under way.

The 359 cultures were isolated from various sources, the majority having been obtained from blood and fecal specimens. Cultures isolated from gall bladder, chest abscess, urine, abdominal wound and river water have likewise been examined. In all cases, the value of the typing method of Craigie and Yen has been evident. Epidemiological studies have repeatedly verified the original investigations of Craigie and Yen regarding the stability of types and the separation of epidemic and endemic cases. Application of the typing results to epidemiological studies is being made as often as is possible and the results show that the typhoid typing method is a valuable and sensitive tool in the control of typhoid fever. Properly applied, the procedure is a useful adjunct to epidemiological studies. Future possibilities in applying the method are dependent on the aid and coöperation of all concerned.

Acknowledgment. Thanks must be expressed to Dr. James Craigie, Connaught Laboratories, University of Toronto, for the original supply of type cultures and homologous bacteriophage preparations. In addition to the aid of the 9 state laboratories concerned, the author wishes to acknowledge the assistance of the San Joaquin Local Health District and the Departments of Health of the County and City of Los Angeles, all of California. Without the whole-hearted coöperation of all the agencies concerned this work would not have been possible.

11697

Carrion's Disease. I. Some Growth Factors Necessary for Cultivation of *Bartonella bacilliformis.*

José F. Jiménez. (Introduced by Roy C. Avery.)

From the Department of Pathology, Vanderbilt University School of Medicine, Nashville, Tenn.

The medium of preference for the isolation of *Bartonella bacilliformis* from the blood of Oroya fever cases and for the routine of stock cultivation is still the one originally used for these purposes by Noguchi: namely, his semi-solid serum agar for leptospira.[1, 2] This medium however, is not suitable for the production of vaccines. In attempts to grow the organism on solid media it soon became apparent that the addition of blood was necessary.[3, 4] It seemed of

---

[1] Noguchi, H., and Battistini, T., *J. Exp. Med.*, 1926, **43**, 851.

[2] Noguchi, H., *J. Exp. Med.*, 1926, **44**, 553.

[3] Aldana, L., *Bacteriologia de la Enfermedad de Carrion Tesis*, Lima, Peru, 1930.

[4] Lawkowic, W., *Trans. Roy. Soc. Med. Hyg.*, 1939, **32**, 601.

interest then, to determine whether one or both the x and v growth factors contained in the blood were necessary for its successful cultivation.

Four strains of *Bartonella bacilliformis* were studied. Two were obtained from Dr. M. Hertig in Peru. They were originally isolated from the blood of 2 fatal cases of Carrion's disease. These strains were pathogenic for monkeys one year after isolation. A third strain was supplied by Dr. D. Weinman of the Harvard Medical School. It had been shown to be pathogenic for the monkey for a period of several years. These 3 cultures were received in Noguchi's medium. A fourth culture was isolated by us from a blood specimen sent by Dr. H. Colichon in Peru, taken from a recovered case of Carrion's disease which no longer showed the organisms in the blood cells. Smears of this blood made in this laboratory were negative. The morphologic, staining and cultural characters of all these strains were similar. None of the strains grew in infusion broth or on agar slants even when heavily inoculated.

As a source of the v factor an aqueous extract of yeast prepared according to Lwoff[5] was used in a concentration of 0.1% in 1% glycerine infusion agar. The x factor was furnished by the addition of 5% defibrinated rabbit's blood which was then autoclaved at 120° C for 20 minutes.[6] Agar plates containing x, v, and xv were inoculated. Cultures of *H. influenzae* and parainfluenza were used as controls on the media. The cultures have been followed for 5 transfers. At each transfer plain infusion broth and agar slants were also inoculated with the bartonella strains and the influenza controls and in each instance no growth occurred. The incubation temperature varied between 25° and 28° C; the pH of the medium between 7.2 and 7.6. Desiccation of the medium was always fatal to growth of this organism. The plates were sealed with rubber.[7] On this medium numerous small translucent colonies of *B. bacilliformis* could be easily seen with the naked eye by the 3rd or 4th day.

The results may be briefly stated as follows: The presence of both factors x and v in the glycerine agar insure an excellent growth for *B. bacilliformis* which becomes apparent on the third day. The medium containing the x factor alone is sufficient to support a good growth in all the transfers but it appeared on or sometime after the fourth day. The basic medium containing the v factor alone is insufficient for successful cultivation. However it is possible to show some restricted growth on the first or second transfers due probably to the x factor carried over with the inoculum. Further transfers on the medium were unsuccessful.

---

[5] Lwoff, A., and Lwoff, M., *Proc. Roy. Soc. London*, 1937, **122**, 352.

[6] Thjotta, T., and Avery, O. T., *J. Exp. Med.*, 1921, **34**, 97, 455.

[7] Petroff, S. A., and Steenken, W., Jr., *J. Exp. Med.*, 1930, **51**, 834.

The small number of experiments with only 4 strains imposes caution in the matter of making definite statements regarding the x and v factors, but it may be inferred from these results that *B. bacilliformis* is incapable of synthesizing the x factor but can produce its own cozymase. This is indicated by the results obtained with the x factor alone. It is interesting in this connection to note that one member of the Hemophilic group, *H. canis,*[8, 9] is similar in this respect to these strains of *Bartonella bacilliformis.*

The macroscopic appearance of *B. bacilliformis* colonies can be of two kinds: 1. Minute, round, mucoid clear colonies which are sometimes confluent; 2. An opaque finely granular yet mucoid film which has a tendency to outgrow the original boundaries of the inoculum. In both types the growth adheres so tenaciously to the medium and to the loop that it is difficult to make a suspension. The suspensions are homogeneous.

On microscopic examination this organism presents great pleomorphism, a character mentioned by many investigators.[10] Mixtures of small bacillary and finely granular shapes sometimes occurring in masses, can be seen. It is not possible at this time to identify the micro- and macro-scopic characters in their relation to the phenomenon of dissociation.

During the experiments on the growth factors v and x, other solid media were tried. Several are worthy of mention since to our knowledge their use for isolation has not been reported in the literature. A blood-glucose-cystine agar gave excellent results. This medium was made by adding 0.1% cystine and 1% glucose to autoclaved beef infusion agar. The medium was then sterilized in flowing steam one hour for 2 days. Defibrinated fresh rabbit's blood was added up to 5% concentration before pouring the plates. One of the strains studied was isolated on this medium directly from the patient's blood specimen. The colonies were numerous, discrete and easily discernible on the third day of incubation. The addition of 0.1% tryptophane did not enhance the growth promoting properties of this medium. Blood glucose agar gave much slower and scantier growth.

---

[8] Rivers, M. T., *Johns Hopkins Hosp. Bull.,* 1922, **33**, 140.

[9] Fildes, P., *Brit. J. Exp. Path.,* 1923, **4**, 265.

[10] Pinkerton, H., and Weinman, D., PROC. SOC. EXP. BIOL. AND MED., 1937, **37**, 591.

Another medium which gave excellent results was a 5% defibrinated rabbit's blood infusion agar to which 1% glycerine had been added. Growth of all the strains on this medium was abundant on the third day. Microscopic examination showed compact masses larger than those seen in the smears from the cystine medium. No changes in the blood pigment were observed in any of the media employed in this investigation.

For comparison it may be stated that a 4 or 5 day growth on either the cystine or glycerine blood agar medium can be favorably contrasted with that obtained in a 15- to 20-day-old culture grown in Noguchi's medium.

Summary. Solid media for the rapid isolation of *Bartonella bacilliformis* are described. It is shown that this organism requires the x growth factor of blood. No growth was obtained with the v factor alone.

11698 P

A New Intravenous Galactose Clearance Test for Differentiation of Obstructive from Parenchymatous Jaundice.*

A. M. BASSETT, T. L. ALTHAUSEN, AND G. COLTRIN

From the Department of Medicine, University of California Medical School, San Francisco.

Galactose is an ideal substance for testing hepatic function because it is utilized exclusively by the liver and because its utilization is independent of insulin. Nevertheless the usual method of performing the galactose test by giving this sugar orally and determining its excretion in the urine gives unreliable results, due in part to wide variations in the rate of intestinal absorption[1, 2] which are especially significant because of the absence of a renal threshold for galactose, and in part to difficulties in measuring the galactose in the urine of patients with marked bilirubinuria. Therefore we have modified the test by administering galactose intravenously and determining its clearance from the blood.

Technique: One cubic centimeter of a 50% solution of galactose per kilogram of weight is injected intravenously. The injection

---

* Aided by a grant from the Christine Breon Fund for Medical Research.

[1] Althausen, T. L., and Stockholm, M., *Am. J. Physiol.*, 1938, **123**, 577.

[2] Althausen, T. L., *Am. J. Digest. Dis.*, 1939, **6**, 544.

should consume 5 minutes. The amount of galactose remaining in the blood 75 minutes after injection is determined by the modified method of Somogyi.[3]

Results. This intravenous galactose tolerance test has been performed on 15 persons with normal livers and on 55 patients with marked jaundice many of whom posed the diagnostic question of obstructive versus parenchymatous jaundice. In the normal group all galactose was removed from the blood in 75 minutes. In most jaundiced patients some galactose was present in the blood at the end of the test, but the amount was strikingly different in the two types of jaundice. (see chart). Of the 34 cases of obstructive jaundice, 28 were confirmed by operation or autopsy, 5 by typical clinical course, and in 1 case the obstruction could not be demonstrated conclusively at operation. Of the 21 cases of hepatitis, 18 were diagnosed by the clinical course and laboratory tests, and 3 by autopsy findings. Patients with acute hepatitis averaged 45 mg % of galactose while those with extrahepatic biliary obstruction of less than 6 months' duration averaged only 14 mg %. If 20 mg % of galactose is chosen as the dividing line, 90% of the cases of hepatitis are above, and 83% of the cases of obstructive jaundice are below this line. In the small group of patients with

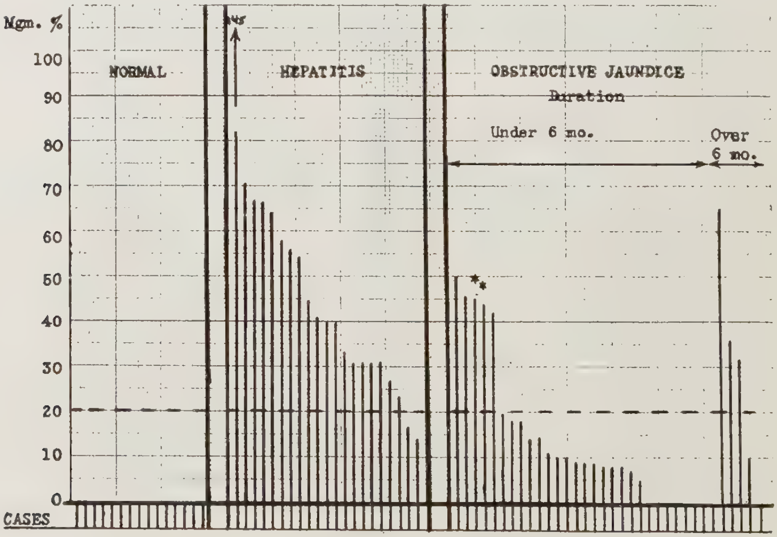


FIG. 1.
Level of galactose in 75-minute blood specimens of individual patients without hepatic disease, with acute hepatitis, and with obstructive jaundice.

---

[3] Raymond, A. L., and Blanco, J. C., *J. Biol. Chem.*, 1928, **79**, 649.

obstructive jaundice of over 6 months' duration, the secondary liver damage robs the test of its value in differential diagnosis. If obstructive jaundice develops in patients with pre-existing parenchymatous disease of the liver, the test shows a confusingly high blood level of galactose. This occurred in 2 cases (identified on the chart by asterisk) in which obstructive jaundice was associated with impaired clearance of galactose. Application of this test to other forms of liver disease is now in progress.

Conclusion. The intravenous galactose tolerance test offers valuable assistance in the differentiation of the parenchymatous or "medical" type from the obstructive or "surgical" type of jaundice.

11699

Antihemorrhagic Activity of Tetra Sodium 2-Methyl-1, 4-Naphthohydroquinone Diphosphoric Acid Ester and Other Naphthoquinone Derivatives.

J. LEE, U. V. SOLMSSEN, AL STEYERMARK AND R. H. K. FOSTER.
(Introduced by E. Chargaff.)
From the Laboratories of Hoffmann-La Roche, Inc.

In a recent communication[1] we reported preliminary data on the antihemorrhagic activity of the tetra sodium salt of 2-methyl-1, 4-naphthohydroquinone diphosphoric acid ester (for brevity, referred to hereafter as substance N-123). At this time preliminary tests indicated that doses of 0.6-0.8 γ administered subcutaneously to chicks on a vitamin K-free diet reduced the clotting time to below 10 minutes. In this report we are presenting more complete observations.

Ansbacher *et al.*[2] recently reported the potency of this phosphoric acid ester to be much less than we indicated. Further work has shown that while occasional chicks require only 0.6-0.8 γ of substance N-123 to reduce the clotting time to 10 minutes or less the average is somewhat higher. This average is still far below that reported by Ansbacher *et al.* Furthermore we find the average dose of methyl naphthoquinone to be higher than reported by the latter authors.[2]

---

[1] Foster, R. H. K., Lee, J., and Solmssen, U. V., *J. Am. Chem. Soc.*, 1940, **62**, 453.

[2] Ansbacher, S., Fernholz, E., and Dolliver, M. A., PROC. SOC. EXP. BIOL. AND MED., 1940, **43**, 652.

Fieser and Fry[3] did not consider the phosphoric acid ester a very potent preparation. They stated it was active at 25 γ but not at 10 γ. The conflict between the results of Ansbacher *et al.* and those of Fieser and Fry on the one hand and ours on the other raises the question of purity of the preparations with which those authors worked. In our experience pure preparations of the tetra sodium salt are only obtained after isolation and purification of the intermediate diphosphoryl chloride and the free diphosphoric acid.

*2-Methyl-*1,4-*naphthohydroquinone-diphosphoryl chloride* is a viscous yellow oil with an odor characteristic for an acid chloride. It cannot be distilled without decomposition.

Analysis:

Found	P 14.46%
Calc. for $C_{11}H_8O_4Cl_4P_2$	P 15.23%

2-Methyl-1,4-naphthohydroquinone diphosphoric acid ester is a pink-tinted, crystalline substance, m.p. 212° (uncorr.) under decomposition.

Analysis:

Found	C 39.75, 39.83%, H 3.65, 3.75%, P 17.87, 17.79%
Calc. for $C_{11}H_{12}O_8P_2$	C 39.52%, H 3.59%, P 18.56%

*Tetrasodium 2-methyl-*1,4-*naphthohydroquinone diphosphoric acid ester* is a colorless crystalline hygroscopic substance containing various amounts of moisture of crystallization depending upon the drying treatment. A typical sample showed after drying to constant weight at 160°C 20.40, 20.60% of moisture (calc. for 5 H_2O, 18.6, calc. for 6 H_2O, 20.3%).‡ The sodium content of the moisture-containing substance was found as 18.02%, 17.02% (calc. 17.95%) and the phosphorus content 12.07%, 11.99% (calc. 12.11%).

For the anhydrous materials the figures correspond fairly closely to the theoretical data.

Calc. for $C_{11}H_8O_8P_2Na_4$	Na = 21.79%, P = 14.69%
Found	Na = 22.10, 20.98%, P = 14.8, 14.7%

Immediately before preparing test solutions the water content is controlled or the solutions themselves are assayed for phosphorus.

In carrying out the test we have adopted an 18 hour curative assay period, a modification of Ansbacher's[4] original method. Day-old white leghorn cockerels were placed on the Almquist[5] diet for 2 weeks. Many chicks developed the characteristic hemorrhages and control samples did not clot in 2 hours. Substances tested were administered subcutaneously or by stomach tube.

---

[3] Fieser, L. F., and Fry, E. M., *J. Am. Chem. Soc.*, 1940, **62**, 228.

‡ The moisture calculated as water. Crystallized from water, methanol and acetone, the moisture probably consists of 2 molecules of methanol and 3 molecules water (theoretical moisture content 21.8%). The material used in these studies was prepared in this manner. Pending definite determinations of the constitution, the substance was referred to as containing the equivalent of 6 molecules of water.

To minimize contamination of the sample by tissue juices, blood samples were taken by brachial venipuncture using a 1 cc tuberculin syringe and a half inch No. 25 needle. 0.25 cc of blood was drawn into the syringe and of this the first 0.20 cc was expressed slowly into a small vaccine tube. The tube was immediately stoppered with a paraffined cork and placed into an automatic tilting rack immersed in a water bath at 38.5°. Coagulation time was determined to the nearest minute, the end-point being taken when the sample ceased to flow.

Because chicks obtained at different times appear to vary somewhat in reactivity we employ a standard of comparison whenever accurate assays are desired. Originally we used a hexane extract of alfalfa as a standard but now employ only methyl naphthoquinone.

As a criterion of potency we employ a modified Thayer-Doisy[6] unit. This unit is defined as the amount of antihemorrhagic substance which will reduce the clotting time of 50% of vitamin K-free chicks to ten minutes or less under our assay conditions. Since it is a curative unit it may be represented by the expression CD50. A 10 minute clotting time, although not normal, is more satisfactory than the normal because it is on a curved portion of the dose-effect curve rather than on the horizontal position where there is little or no change in clotting time with change in dosage.

The method of determining the CD50 has been to inject 2 groups of chicks subcutaneously at different dose levels and plot the doses against the per cents of samples clotting at 10 minutes or less on logarithmic probability paper. The CD50 is that point where a straight line drawn between the two data crosses the 50% probability coördinate.

In Table 1 are given 3 sets of assays on substance N-123. The ratios of potency between N-123 and methyl naphthoquinone are 0.52, 0.44 and 0.47. Similar values have been observed in many other assays. The average CD50 for methyl naphthoquinone in 7 different assays was 0.64 γ with extremes of 0.61 and 0.75 γ. This dose only brings the average clotting time to 10 *minutes*

---

[4] Ansbacher, S., *Science*, 1938, **88**, 221.

[5] Almquist, H. J., *J. Biol. Chem.*, 1936, **114**, 241.

[6] Thayer, S. A., MacCorquodale, D. W., McKee, R. W., and Doisy, E. A., *J. Biol. Chem.*, 1938, **123**, cxx.

TABLE I.

Bioassay of Sodium 2-methyl-1,4-naphthohydroquinone Diphosphoric Acid Ester ($MeNHQ$ Phos · $6H_2O$) and 2-methyl-1,4-naphthoquinone (MeNQ). Injections made subcutaneously using 0.2 cc of aqueous solutions.

Chick Group No.	Substance	N*	CD50	Ratio of Potencies
			γ	
505-506	MeNHQ Phos · $6H_2O$	24	1.31	.47
507-508	MeNQ	21	0.61	
542-543	MeNHQ Phos · $6H_2O$†	28	1.25	.52
540-541	MeNQ†	27	0.65	
544-545	MeNHQ Phos · $6H_2O$	37	1.65	.44
546-547	MeNQ	40	0.72	

*Divided about equally between the two dose levels.

†The actual doses injected were 0.5 and 1.0 γ per chick for MeNQ and 1.0 and 2.0 γ per chick for MeNHQ Phos · $6H_2O$.

whereas Ansbacher reported that 0.25 γ brings the average clotting time to *normal*. In his earlier reports he stated the activity to be 0.5 γ, which is more nearly in line with our figures. The average of 8 assays on substance N-123 is 1.34 γ per CD50 and the potency relative to methyl naphthoquinone as determined by these averages is 0.48.

In Table II are listed assays comparing oral and subcutaneous potencies of the 2 substances. Fewer animals were used in these tests so obviously the standard errors must be greater. Approximations of CD50's were obtained by drawing the best fitting lines on logarithmic probability paper, parallel to the average slope of many other assays. The oral CD50's were found to be 1.9 γ for substance N-123 and 1.5 γ for methyl naphthoquinone. The relative

TABLE II.

Comparison of Potencies After Oral and Subcutaneous Administration. (Assays conducted simultaneously.)

Chick group No.	Substance	Dose per chick	N	Route	Avg clotting time	% of samples clotting at 10′ or less
		γ			min	
553	MeNHQ Phos · $6H_2O$[4]	2	8	P.O.	14.6*	63
554	”	4	8	”	8.0	88
556	MeNQ	1	8	”	18.1*	12
557	”	2	6	”	6.5	100
552	MeNHQ Phos · $6H_2O$[4]	2	7	S.C.	12.8	43
555	MeNQ	1	7	”	10.3	57

*In both instances 2 samples had not clotted at 30 minutes. For purpose of averages, these samples were treated as though they had clotted at 30 minutes.

TABLE III.
Results on Other Naphthoquinone Derivatives.
Oral administration of 0.2 in sesame oil. Eighteen-hour assay method.

Substance	Dose γ/chick	N	Clotting time
			min
2-n-butyl-3-hydroxy-1,4-naphthoquinone	1000	3	>30
2-allyl-3-hydroxy-1,4-naphthoquinone	1000	3	>30
3,4-dihydroxy-1,2-naphthoquinone (isonaphthazarine)	200	3	>30
γ,γ-dimethyldihydropyrano-1,4-naphthoquinone (α-lapachone)	200	3	>30
β,β-dimethyldihydrofurano-1,4-naphthoquinone	200	3	>30

strength of the former is thus 0.79. The subcutaneous assays in this series gave 2.2 γ and 0.9 γ respectively with a potency ratio of 0.41.

Ansbacher found the activity ratio by oral or intravenous administration between the phosphoric acid ester and methyl naphthoquinone to be about 1:20. Our data show this ratio to be slightly less than 1:2 by subcutaneous administration and our oral tests, although limited in number, indicate that the potency ratio must be far closer to 1:2 than 1:20.

In Table III are listed the bioassay results of 5 naphthoquinone derivatives not heretofore reported in the literature. These samples were prepared by one of us (A.S.) in the laboratory of the late Dr. Samuel Hooker.[7] We made no attempt to determine what the active dose was, if any, all substances being found inactive at the comparatively high doses studied. Neither the furano- nor the pyrano-naphthoquinones tested showed any activity, possibly due to the presence of the oxygen linkage in position 3. Isonaphthazarine (3,4-dihydroxy-1,2-naphthoquinone) was also tested in order to determine the effect of o-quinones on the activity, although it was known that vitamin K must be a p-quinone since it is yellow.[7]

Discussion. Substance N-123 used in these experiments contained the equivalent of 6 molecules of water of crystallization* which amounts to 20.2% of its weight, the total molecular weight being 530. The molecular weight of methyl naphthoquinone is 172 and the ratio 172:530 is 0.325. The average ratio of potency was found to be 0.48, however, so evidently the phosphoric acid ester is more active, molecule for molecule, than methyl naphthoquinone. This higher molecular activity is in the proportion of 0.48 to 0.325

---

[7] Hooker, S. C., and Steyermark, Al, *J. Am. Chem. Soc.*, 1936, **58**, 1163.

* The substance is hygroscopic and may even liquefy if the humidity is high. See also footnote ‡.

or 1.48, that is, substance N-123 is about 50% more active than methyl naphthoquinone on a molecular basis.

Summary. 1. The CD50 (Thayer-Doisy unit) by subcutaneous administration of tetra sodium-2-methyl-1,4-naphthohydroquinone diphosphoric acid ester ($C_{11}H_8O_8P_2Na_4 \cdot 6H_2O$)[4] is 1.34 γ. 2. The subcutaneous CD50 of 2-methyl-1,4-naphthoquinone ($C_{11}H_8O_2$) is 0.64 γ.

3. The ratio of these potencies is 0.48 but on a molecular basis allowing for 6 molecules of water of crystallization the phosphoric ester is 50% more potent than methyl naphthoquinone.

4. Five naphthoquinone derivatives not hitherto reported are shown to be inactive in doses from 200 to 1000 γ.

11700

Pharmacological Observations on Tetra-Sodium-2-Methyl-1, 4-Naphthohydroquinone Diphosphoric Acid Ester.

R. H. K. FOSTER. (Introduced by E. Chargaff.)
(With the technical assistance of H. H. Clark.)
From the Pharmacology Laboratory of Hoffmann-La Roche, Inc.

The antihemorrhagic activity of tetra-sodium-2-methyl-1,4-naphthohydroquinone diphosphoric acid ester ($C_{11}H_8O_8P_2Na_4 \cdot 6\ H_2O$)* referred to as N-123 hereafter) has been reported elsewhere.[1, 2] Molecule for molecule it was found to be about 50% more potent than methyl naphthoquinone. In this report we wish to give the general pharmacological properties.

Fatal Dose. Molitor and Robinson[3] reported the LD50 of 2-methyl-1,4-naphthoquinone as 500 mg/kg orally in mice and 75 mg/kg intraperitoneally. In 19-day chicks they found the intraperitoneal dose to be $<$ 100 mg/kg. If their figures of 70% mortality for 100 mg and 90% mortality for 150 mg are plotted on logarithmic probability paper the LD 50 is calculated to be 76 mg/kg.

Although our routes of administration were such that a direct

---

* See footnote ‡ of previous paper.

[1] Foster, R. H. K., Lee, J., and Solmssen, U. V., *J. Am. Chem. Soc.*, 1940, **62**, 453.

[2] Lee, J., Solmssen, U. V., Steyermark, Al, and Foster, R. H. K., PROC. SOC. EXP. BIOL. AND MED., 1940, **45**, 407.

[3] Molitor, H., and Robinson, H. J., *ibid.*, 1940, **43**, 125.

TABLE I.
Toxicity Studies on Tetra Sodium 2-methyl-1,4-naphthohydroquinone Diphosphoric Acid Ester.

Animal	Route of administration	N	LD50 mg/kg
Mouse	I.V.	50	450
"	S.C.	40	450
Chick (normal diet)	"	20	675
Rabbit	I.V.	2	>100 and <200
Cat*	"	1	690 (divided doses)

*Circulatory experiment under Alurate anesthesia.

comparison between methyl naphthoquinone and substance N-123 is not possible, the following data illustrate the obviously lesser toxicity of the latter substance. In mice we found the intravenous LD50 to be 450 mg/kg and the subcutaneous LD50 was likewise 450 mg/kg.

Accurate toxicities noted above for substance N-123 were determined only in mice. Using 20 normal chicks the subcutaneous LD50 was estimated at 675 mg/kg. One rabbit died with an intravenous dose of 200 mg/kg, whereas one receiving 100 mg/kg recovered with no severe symptoms. All toxicities are listed in Table I. The fatal doses are far beyond the antihemorrhagic doses. In chicks the ratio of the antihemorrhagic CD50 to the LD50 is about 1:30,000.

Toxic Signs. One rabbit was given 50 mg/kg intravenously and this caused an acceleration of respiration, marked constriction of the pupils, increased alertness and a rise in temperature of the ears. The pupils were normal in 15 minutes though the respiration continued rapid for some time. After 100 mg/kg intravenously in another rabbit there was a more marked increase in alertness, the animal moving about the cage with some agitation and showing signs of anxiety or apprehension. In 3 or 4 minutes it became quiet and crouched in one corner of the cage. The pupils were pin point in size and remained so for an hour, becoming normal at the end of 2 hours. A third rabbit received 200 mg/kg intravenously and showed greater agitation than the previous rabbit (but still much less than, for example, the violent morphine excitement in cats). Seven minutes after injection the animal screamed. The screaming lasted about 15 seconds and was repeated a few minutes later. At 30 minutes after injection a convulsion occurred and at 35 minutes after injection the animal died. Respiration was not accelerated as with the smaller doses but remained slow and labored throughout with the animal in prostration after the first few minutes. Considerable salivation occurred. There were no particular findings at autopsy except a generalized moderate congestion and a darkening of arterial blood indicating death by respiratory paralysis.

In chicks the outstanding signs were the panting type of respiration with the beaks open, pupillary constriction, preliminary mild excitement or anxiety, later prostration and death. Convulsions did not occur. In mice the syndrome was essentially the same as in chicks and rabbits, though the preliminary excitement and stimulation was usually somewhat greater. Occasional convulsions occurred.

Circulation and Respiration. In cats anesthetized with Alurate* there was a rise in both systolic and diastolic blood pressure amounting to as much as 50-60 mm mercury following the administration of 5 or 10 mg/kg. Subsequent doses caused a somewhat diminishing response. The rise in blood pressure was invariably well sustained with return to normal occurring only after 10 or 15 minutes or more. The pulse rate was generally stimulated from a slight to moderate extent, in one case corresponding from 170 to 220 per minute, this being about the extreme.

The effect on respiration was marked, 10 mg/kg often causing violent respiratory activity both in rate and depth. This usually diminished after two to five minutes to somewhat less than normal and later recovered. Following two or three preliminary small doses one cat was given 50 mg/kg every 5 minutes until death. The total amount administered was 690 mg/kg.

In cats under ether the effect on blood pressure was less than when under Alurate anesthesia. The pulse was stimulated slightly and the respiration showed as high a degree of stimulation as under Alurate.

Another type of respiratory experiment was tried in which rabbits received 10 mg/kg morphine sulfate. A head mask connected with a spirometer was employed so that minute volume of respired air could be measured. Ten and 20 mg/kg had substantially no effect and 50 mg/kg caused but a slight increase in minute volume and in rate. There was later a decrease in both minute volume and rate.

Irritation. In no case was any sign of irritation noticed after subcutaneous injection of a 5% solution in guinea pigs in doses of 50 to 150 mg/kg or in chicks or mice surviving doses in the lethal range.

Discussion and Summary. Although from the standpoint of treating prothrombin deficiencies lethal and sublethal doses of tetrasodium-2-methyl-1,4-naphthohydroquinone diphosphoric acid ester are without practical significance, it is interesting to note their effects on the central nervous system. It seems likely that most of the peripheral effects observed are the result of central action but complete analysis of the manner of action has not yet been made. The anesthetic employed appears to possess a quantitative influence on the circulatory but not so much on the respiratory action. With ether anesthesia in cats there is less hypertension after substance N-123 than with Alurate anesthesia. The substance had very little influence on respiration in morphinized rabbits, but in cats under Alurate or ether anesthesia there was marked respiratory stimulation. As is true with most substances acting as biological catalysts (enzymes, vitamins, hormones) the safety margin of its antihemorrhagic effect is enormous. All doses are for the crystalline product containing a moisture equivalent of 6 molecules of water.

---

* Allyl-isopropylbarbituric acid.

11701

Effects of Spinal Anesthesia upon Venous Pressure in Man.

JOHN ADRIANI AND E. A. ROVENSTINE.
(Introduced by Stevens J. Martin.)

From the Division of Surgery, Department of Anesthesia, New York University College of Medicine, New York City.

The fall in arterial pressure accompanying high spinal anesthesia in man has been widely investigated. Although paresis of sympathetic fibres with dilation of the arterioles has been regarded as the mechanism for the fall, a recent report by Smith and co-workers[1] indicates an autonomous control of the arterioles of the viscera. They postulate that circulatory failure is primarily venous failure. A fall in venous pressure would be expected. Venous pressures during spinal anesthesia have been reported by Schuberth[2] and Seevers,[3] Goldfarb, *et al*,[4] but the observations have been either on animals or isolated readings in the course of anesthesia for man and there is no agreement among these authors. No studies have been found that correlated arterial tension, with venous pressure and the extent or height of anesthesia. This investigation was undertaken to supply these data.

---

[1] Smith, H. W., Rovenstine, E. A., Goldring, W., Chasis, H., and Ranges, H. A., *J. Clin. Invest.*, 1939, **18**, 319.

[2] Schuberth, O. O., *Acta Chir. Scandinav.*, 1936, 78 supp., **48**, 1.

[3] Seevers, M. H., and Waters, R. M., *J. A. M. A.*, 1932, **99**, 961.

[4] Goldfarb, W., Provisor, B., Koster, H., *Arch. Surg.*, 1939, **39**, 429.

Procedure. Twenty normal male subjects undergoing extra abdominal operations in the supine position were used to compile these data. Anesthesia was induced by a standard technic using 150 mg of a 10% solution of procaine in spinal fluid. It was injected in the second lumbar interspace. Levels of sensory anesthesia were deter-

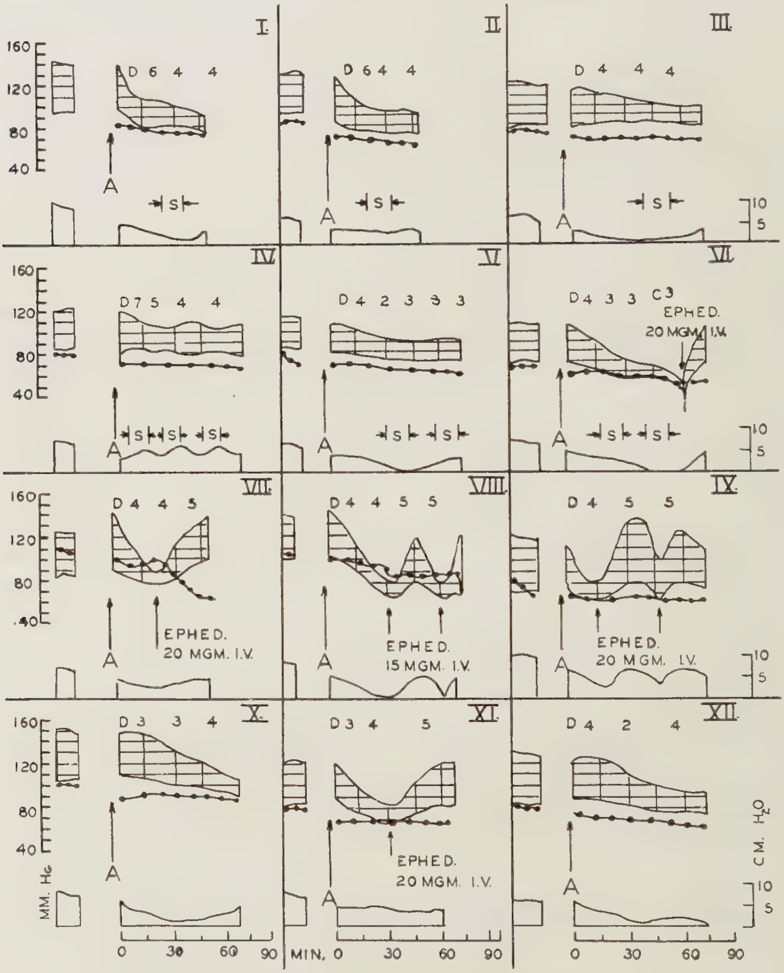


PLATE I.

The blood pressure in mm Hg (upper graph), the pulse rate (dotted line), and the venous pressure in cm H_2O (lower graph) during spinal anesthesia in man. The height of sensory anesthesia is indicated by D_6–, D_5–, etc., at the top of each figure. A– indicates induction of anesthesia and ←S→ period of faradic stimulation to large muscles.

mined according to the classification of Foerster.[5] Direct venous pressure measurements were taken from veins in the antecubital fossa using a modified Moritz-Tabora technic which allowed minute to minute observation throughout the course of anesthesia. Control readings were recorded for a 15 minute period prior to induction. Arterial pressures and pulse rates were also noted.

Results. In 12 subjects anesthesia extended to or beyond D_4 and was accompanied by intercostal paralysis (Plate 1). In 8 subjects it was below this level and accompanied by intercostal activity. The latter were considered as low spinal anesthesias. A consistent immediate fall in venous pressure accompanied high spinal anesthesia amounting to 25% of the control level. Decreases in the arterial pressure were accompanied by further lowering of venous pressure which paralleled arterial pressure fall. In 4 instances the pressure reached zero. (Fig. 5, 6, 8, 12). In low spinal anesthesia the changes in venous pressure were inconsistent. No changes were recorded in 3, there was a rise in 2 and a fall in the remaining 3. In 4 of 5 cases in which intravenous ephedrine was given (Fig. 6, 7, 8, 9, 11), there was a parallel rise in venous pressure and blood pressure. The fluctuations of venous pressure normally seen with respirations were absent or markedly decreased in excursion during anesthesia.

The effects of stimulating muscles of the lower extremity were observed by supplying a faradic current interrupted 30 times per minute, to moist foil electrodes wrapped about the legs and lower abdomen.[6] No significant rises in arterial or venous pressures were obtained in 5 of 6 experiments. (Fig. 1, 2, 3, 4, 5, 6). These results are not in accord with the expression of Ornstein on the value of faradic stimulation of large muscles to counteract decreased arterial tension.[6]

Summary. A decrease of venous pressure follows spinal anesthesia accompanied by intercostal paralyses irrespective of arterial pressure changes. A further decrease accompanies a fall of arterial pressure. No consistent changes accompanied low spinal anesthesia.

---

[5] Foerster, O., *Brain,* 1933, **56**, 1.

[6] Ornstein, G., *Anes. and Anal.,* 1940, **19**, 157.

11702 P

Acquisition of Conditioned Reactions to Angular Acceleration After Cortical Lesions.

M. J. OPPENHEIMER AND E. A. SPIEGEL.*

From the Department of Physiology and the Department of Experimental Neurology, D. J. McCarthy Foundation, Temple University, School of Medicine, Philadelphia, Pa.

Conditioned reactions (CR) to angular acceleration and discrimination of the direction of the accelerated rotation were previously demonstrated (Spiegel and Oppenheimer[1]). Experiments on labyrinthectomized dogs indicated that discrimination of direction of rotation in the horizontal plane at low acceleration is an important criterion of the analyzing function of the labyrinth. Since the changes in the electro-corticogram produced by stimulation of the labyrinth (Spiegel,[2] Kornmueller,[3] Price and Spiegel,[4] Gerebetzoff[5]) suggest that labyrinthine impulses reach the cerebral cortex, it seemed of interest to ascertain to what extent cortical centers participate in the development of these reactions, and the ability of dogs with cortical lesions (2 frontal, 2 parietal and 2 temporal lobectomies) to acquire these CR was studied. One frontal and both parietal lobectomies were performed in one stage, one frontal and both temporal lobectomies in two stages. 14-51 days after the last operation conditioning was started. The dogs were rotated as previously[1] described in clockwise or counterclockwise direction first at slow constant speed which later passed smoothly into accelerated rotation. During the counterclockwise accelerated rotation the unconditioned stimulus (electric shock) was applied, while it was omitted during clockwise rotation. The reactions of the leg and the respiration were recorded. The animals were usually tested every 2nd or 3rd day, but in some cases longer rest periods (up to several weeks) were intercalated.

In both dogs with bilateral ablation of the *parietal lobes* CR to angular acceleration were obtained in the first test series; discrimination of direction developed on the third day of experimentation

---

* Aided by a grant of the National Research Council to E. S.

[1] Spiegel, E. A., and Oppenheimer, M. J., *Am. J. Physiol.*, 1939, **125**, 265.

[2] Spiegel, E. A., *Arch. Neur. and Psych.*, 1934, **31**, 469.

[3] Kornmueller, A. E., *Bioelektr. Erscheinungen d. Hirnrindenfelder*, Leipzig, Thieme, 1937.

[4] Price, J. B., and Spiegel, E. A., *Arch. Otolaryngol.*, 1937, **26**, 658.

[5] Gerebetzoff, M., *C. r. Soc. de biol.* (Soc. belge de biol.), 1939, **131**, 807; *Arch. internat. de physiol.*, 1940, **50**, 55.

in dog No. 506 in which conditioning was started 26 days after bilateral parietal lobectomy and somewhat later in dog No. 486 (inconstant discrimination on the fifth day, constant discrimination on the seventh day); in this dog conditioning was started 17 days after operation. After bilateral 2-stage *frontal* lobectomy (No. 593) the CR as well as discrimination were obtained in the first series of experiments, while the dog operated in one stage (No. 553) developed the CR as well as the discrimination later (on the third day conditioned respiratory reactions, on the sixth day of training conditioned leg reactions and on the same day differentiation of direction). Since the training of both animals started at the same interval (17 days) after the bilateral frontal ablation was completed, it seems that the learning abilities were less impaired by the 2-stage than by the one-stage operation. After the bilateral *temporal* lobectomies we see again a certain influence of the interval between the second operation and the beginning of the training, the animal with the longer interval (No. 584, 51 days) developing the CR in the first series of tests, the animal with the shorter interval (No. 554, 14 days) showing the first CR not before the fourth day. Discrimination of direction appeared in both animals relatively late (on the eighth day of training in No. 584, on the ninth day in No. 554).

Thus bilateral ablation of the frontal, parietal or temporal lobes does not prevent the development of CR to angular acceleration, and such reactions may appear in favorable cases without definite delay compared with normal animals. Discrimination of direction may also be obtained after these operations; there is however usually a delay in its development; only one animal (2-stage frontal lobectomy) showing discrimination on the first day of testing. This delay is the longest after ablation of the temporal lobes, discrimination appearing at the eighth and ninth test series respectively. If we compare the delay after temporal ablations with the longest delay after frontal and parietal lobectomies (Nos. 553 and 486), we have to bear in mind that in these latter animals the bilateral operations were performed in one stage, while the temporal lobectomies were 2-stage operations. Furthermore training was started in No. 553 and 486 17 days after operation, while it was started in one temporal lobectomy (No. 584) 51 days after the last operation. Thus the animals with temporal lobectomies were under more favorable conditions than the frontal lobectomy No. 553 and the parietal lobectomy No. 486 as regards operation in stages and No. 584 also as regards interval between last operation and beginning of the training. As quick a learning of discrimination of direction could have been expected in the animals with temporal lobectomies as in the most favorable cases of frontal and parietal lobectomies, if functional differences between the lobes played no role. It is therefore perhaps not without significance that the longest delay in development of discrimination of direction was observed after the temporal lobectomies.

For an evaluation of these observations it may be recalled that a postoperative development of simple CR to sound and light stimuli is possible after decortication or after ablation of the cortical endings of the respective sensory systems and that the acquisition of such reactions is not or only slightly delayed.[6] As pointed out by Jacobsen[7] the fact that such CR may be mediated through subcortical mechanisms in absence of the cerebral cortex does not exclude the possibility that the cortex plays a role in these reactions in the intact brain, as shown by experiences on post-operative retention and forgetting of CR in cortical lesions. Experiments in this direction with CR to labyrinthine stimuli will be reported later.

11703

Effect of Denervation of Intestine on its Motor Responses at a Site of Distention.

W. B. YOUMANS.

From the Department of Physiology, University of Oregon Medical School, Portland.

The inhibition of motility at sites between 3 and 20 cm in each direction from a region of distention in the innervated and denervated intestine of unanesthetized dogs has been described.[1] The following is an analysis of responses of the small intestine at the site of distention.

Methods. Sixteen dogs with Thiry or Thiry-Vella fistulae of the jejunum as follows were used: 7 with innervated intestinal segments, 2 with both innervated and denervated intestinal segments, 3 with innervated segments that were subsequently denervated, and four with denervated segments only. Several of these animals were the same ones that were used in the former study. Intestinal motility was recorded by a balloon-mercury-manometer system in which the balloon and pressure tubing connecting the balloon with the manometer were filled with water.

---

[6] Poltyreff, S. S., and Zeliony, O. P., *Z. f. Biol.*, 1930, **90**, 157; Culler, E., and Mettler, F. A., *J. Comp. Psychol.*, 1934, **18**, 291; Marquis, D. G., and Hilgard, E. R., *J. Comp. Psychol.*, 1936, **2**, 157; *Brain*, 1937, **60**, 1; Kluever, H., *J. Psychol.*, 1936, **2**, 49; 1937, **4**, 383.

[7] Jacobsen, C. F., *Physiol. Rev.*, 1939, **19**, 303.

[1] Youmans, W. B., Meek, W. J., and Herrin, R. C., *Am. J. Physiol.*, 1938, **124**, 470.

Execution of an experiment was as follows. The balloon was emptied by gravity through a side-arm attached to the pressure tubing, and adjustments were made so that the manometer recorded the zero pressure level. The empty balloon was then inserted into the fistula of the unanesthetized animal. Several minutes later 7 cc of water were introduced into the side-arm of the manometer system as rapidly as possible from a 10 cc syringe. This procedure produced

TABLE I.

Pressures Attained (in mm of Hg.) Following Sudden Filling of Innervated and Denervated Intestinal Segments in Intact Dogs.

Experiment No. and dog No.	Maximum pressure at end of filling	Active increase in response to filling	Absolute maximum if active increase occurred	"Diastolic" pressure at end of 15 min
		Innervated segments.		
I, 1	118			42
II, 1	142			36
III, 1	124			54
IV, 1	128			54
V, 1	140			32
VI, 1	162			42
VII, 1	130			34
VIII, 1	130			22
IX, 1	114			44
IX, 2	118			44
X, 1	76			36
X, 2	126			36
XI, 1	162			68
XI, 2	110			56
XI, 3	140			60
XII, 1	168			70
Avg	130			46
		Denervated segments.		
VII, 2	170			28
VII, 3	164			30
VIII, 2	144	74	218	58
X, 3	124	20	144	46
X, 4	122			44
X, 5	120			50
XI, 4	160	8	168	92
XI, 5	148	14	162	80
XIII, 1	180	44	224	98
XIV, 1	172			80
XIV, 2	220			91
XV, 1	164	50	214	70
XV, 2	190			74
XVI, 1	158			82
Avg	160			66

a sharp rise in pressure as seen in the illustration. The balloons were of sufficient size to accommodate the amount of fluid used without being stretched. Records were analyzed with regard to (1) pressure at the end of the introduction of the water, (2) maximum pressure attained, and (3) "diastolic" pressure at the end of 15 minutes.

Results. The responses of the denervated segments of intestine were found to differ from the innervated with regard to (1) resistance to stretch, (2) excitatory responses to distention, and (3) rate of decrease of the pressure during the sustained distention. Analyses of individual experiments are shown in the table. In innervated intestinal segments the pressure at the end of the introduction of the water into the manometer system ranged from 76 to 168 mm of Hg. with an average of 130 mm of Hg. In the denervated segments the pressures reached at the end of the introduction of the water ranged from 120 to 220 mm of Hg. with an average pressure 160 mm of Hg. The increased resistance to stretch in the denervated intestine may be explained as a true increased tonus of the smooth muscle or as a result of atrophic and fibrotic changes which occur in the unexercised intestine after denervation. An attempt was made to minimize the latter factor by the use of freshly denervated and exercised

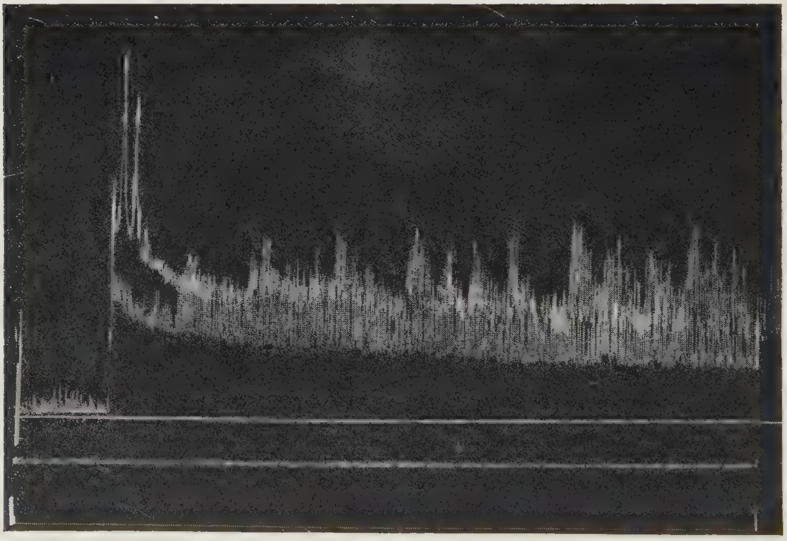


FIG. 1.

Superimposed curves showing differences in pressure changes in the innervated (lower record) and denervated (upper record) intestinal segments of the same unanesthetized dog produced by rapid filling with identical volumes of water. Balloon-mercury-manometer records. Time in 5-second and 1-minute intervals. Base line indicates zero pressure. Further explanations are given in text.

segments. The increased resistance to stretch following denervation was evident in some cases when the segment was used within one week following the operation.

A direct stimulatory effect of distention on the smooth muscle of the intestinal wall was demonstrated. In the innervated intestine the maximum pressure resulted from the resistance to stretch and was attained at the end of the filling with water. This is illustrated in the lower record of the figure. In 6 of the experiments the denervated intestinal smooth muscle responded to the filling with strong contractions so that the pressure was actively increased to still higher levels. This type of response is illustrated in the upper record of the figure. In three instances pressures of 214 to 224 mm of Hg. were produced by the contraction of extrinsically denervated intestinal smooth muscle against the contents of the distending balloon. The high pressures thus produced lasted for less than a minute and were followed by a sharp reduction in pressure. Gradual reduction in pressure continued during the remainder of the 15 minute period.

At the end of 15 minutes the "diastolic" pressure in the innervated intestine was between 22 and 70 mm of Hg. with an average of 46 mm of Hg., while in the denervated intestine it was 28 to 98 mm of Hg. with an average of 66 mm of Hg.

Failure of the innervated intestine to show the strong motor response to distention which commonly occurs in the denervated intestine affords evidence that the innervated intestine at the site of distention is subject to inhibitory influences from extrinsic nerves.

Interpretation. The combined results of this and of the previous study[1] provide a basis for description of the sequence of responses of the innervated and the denervated intestine to rapid filling. The pressure or stretch produced by distention stimulates afferent nerve endings, and reflexes are competed over extrinsic nerves rapidly resulting in inhibition of the intestinal smooth muscle along a considerable section including the site of the distention. Denervation of the intestine unmasks a direct excitatory effect of distention on the smooth muscle at the site of distention. A result of these responses is hypermotility of the intestine at the site of distention as compared with the motility in immediately adjacent segments. Therefore, the extrinsic nerves of the intestine contain the pathways for reflexes having a contents-dispersing and pressure-regulating value. Distribution of contents is accomplished in the denervated intestine by the development of relatively higher pressures at the site of distention and by more slowly acting inhibitory influences mediated by local mechanisms.

Summary. The role of the extrinsic nerves in the local intestinal response to filling and distention has been studied by subjecting innervated and denervated intestinal segments of unanesthetized dogs to identical distending forces. Distention acts as a stimulus to the smooth muscle of the denervated intestine so that an active pressure increase may be induced by the stretch and passive pressure resulting from a standard amount of filling. The stimulatory effect of filling is not equally evident in the innervated intestine. This result may be interpreted as indicating that the degree of motility observed at the site of a distention in the innervated intestine depends, in part, upon a balance between direct stimulatory effects and reflex inhibitory effects.

11704

Cobalt Color Reaction of Barbiturates.*

Richard F. Riley, R. F. Krause, Luville T. Steadman,
F. E. Hunter and Harold C. Hodge.

From the Departments of Biochemistry and Pharmacology, and Radiology, School of Medicine and Dentistry, The University of Rochester, Rochester, N. Y.

Of the numerous analytical methods employed for the determination of barbiturates, those making use of specific color reactions have been most extensively studied. Handorf[1] employed the murexide reaction for the detection of veronal, and Zwikker,[2] Bodendorf,[3] Herwick[4] and Koppanyi, *et al.*,[5, 6] have developed modifications of a color reaction with cobalt salts in conjunction with various bases.

Kozelka and Tatum[7] attributed this color formation to a specific reaction with substances containing one or 2 imide groups. In an investigation of amytal (isoamylethyl barbituric acid) excretion by dogs, using a modification[9] of the reaction designed by Koppanyi

---

* This work was supported in part by grants from the Committee on Therapeutic Research of the Council on Pharmacy and Chemistry of the American Medical Association, and from the Carnegie Corporation of New York.

The authors thank the Eli Lilly Company, especially M. M. Weaver, for providing the amytal and the degradation products of amytal used in this study.

[1] Handorf, H., *Z. f. d. ges. exp. Med.*, 1922, **28**, 56.

[2] Zwikker, J. J. L., *Pharm. Weekblad.*, 1931, **68**, 975.

[3] Bodendorf, K., *Arch. Pharm.*, 1932, **270**, 290.

[4] Herwick, R. P., *Arch. internat. de Pharm. et de Therap.*, 1933, **45**, 160.

[5] Koppanyi, T., *et al.*, *ibid.*, 1933, **46**, 76.

[6] Koppanyi, T., *et al.*, *Am. Pharm. Assn.*, 1934, **23**, 1074.

[7] Kozelka, F. A., and Tatum, *J. Pharm. and Exp. Therap.*, 1937, **59**, 54.

[9] Krause, R. F., and Riley, Richard F., unpublished work.

et al.,[6] employing cobalt acetate and isopropylamine made up in absolute methanol solutions, it was observed in this laboratory, that of the more probable breakdown products of amytal[8] only isoamylethyl acetic acid gave the characteristic color reaction. Since this compound contains no imide grouping, it was apparent in this case that color formation was not due to such a group.

This finding led to an examination of various classes of compounds a number of which have not been reported upon by other investigators. A partial listing is given in Table 1.

Since the acids tested gave a color apparently identical with that obtained with amytal, a number of these test solutions were submitted to spectrophotometric analysis.

The spectrophotometric curves given herewith were made with a Bausch and Lomb medium quartz spectrograph and sector photometer. The length of the absorption cells was 5 cm. To cobalt acetate and isopropylamine mixtures were added respectively, solutions of amytal, isoamyl ethyl acetic acid ($RRC_2H_2O_2$) and stearic acid in amounts sufficient to give an intensity of color suitable for measurement.

It is noted that the addition of isopropylamine to the cobalt acetate produces a shift of the absorption band from 5150 to 5450 AU, and then for the other 3 compounds there is a further shift for each to 5650 or 5700 AU. It is also interesting to note that the absorption

TABLE I.
Compounds Tested for Color Reaction.

Compound tested	Results	
Acetic acid	+	Compounds soluble in chloroform and possibly present in urine specimens
Propionic acid	+	
Butyric acid	+	
Isoamyl-ethyl acetyl urea	—	
Isoamyl-ethyl acetamide	—	
Isoamyl-ethyl acetic acid	+	
Hippuric acid	+	Compounds insoluble in chloroform and possibly present in urine specimens
Creatinine	+	
Uric acid	—	
Allantoin	—	
Histidine	—	
Theobromine	—	
Caffeine	—	
Diphenyl amine	—	Miscellaneous compounds
Benzoic acid	+	
Benzaldehyde	+	
Heptaldehyde	+	
Stearic acid	+	
Methyl propionate	—	

---

[8] Shonle, H. A., *et al.*, *J. Pharm. and Exp. Therap.*, 1933, **49**, 393.

maxima exhibited by the 2 acids and by amytal are nearly the same. This may indicate that the colored substances formed are of similar structure in the 3 cases. This together with the fact that all 3 are acidic compounds with ionizable hydrogen atoms may be considered presumptive evidence for salt formation as the mechanism of this reaction.

The following scheme was employed in the development of the cobalt color for quantitative measurement. To 0.2 ml of 1% cobalt acetate in absolute methanol, in a colorimeter cup, are added in the following order: 0.6 ml of 5% isopropylamine in absolute methanol, 2.0 ml of chloroform, and 1.0 ml of the chloroform extract. The solutions were well mixed by rotation, and the intensity of the color produced read in terms of an arbitrary scale on a photoelectric colorimeter.

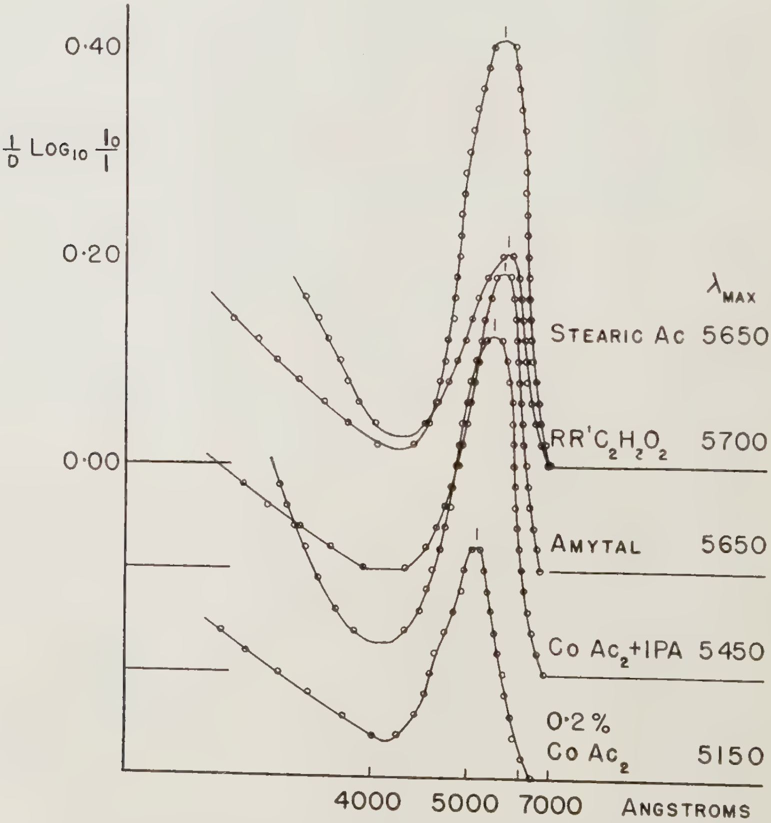


FIG. 1.

Spectrophotometric curves of cobalt acetate and cobalt acetate plus isopropylamine and these reagents plus amytal, isoamyl ethyl acetic acid ($RR^1C_2H_2O_2$), and stearic acid.

Using this technique, chloroform extracts of urine that contain no barbiturates have been examined. It was found that the developed color showed a maxima of 5600 and 5750 AU, for copper sulfate treated[6] and untreated urine, respectively. We have found that such extracts contain chromogenic substances[9] which may possibly be of acidic character. Inasmuch as these factors produce similar absorption spectra, and can give artifactual excretion values, results obtained using the cobalt acetate and isopropylamine test in the study of barbiturate excretion in urine must be considered cautiously and accepted with reservation.

11705

Variations in Concentration of Certain Electrolytes of Blood Serum During Induced Hyperpyrexia.

W. D. PAUL AND C. R. KEMP.

From the Department of Internal Medicine and Pathological Chemistry Laboratory, State University of Iowa, College of Medicine, Iowa City, Iowa.

The remarkable constancy of the potassium content of the blood under pathologic conditions has been long recognized, but recent studies have brought forth evidence that this constancy may be disturbed greatly in cases of shock of various kinds. However, no uniformity of opinion has emerged from studies of the potassium fluctuations in shock. Different workers have reported different changes in the potassium content of the blood in cases of induced shock, and a variety of conclusions as to the role which such variations play has been drawn. The question whether variations in the blood potassium may be the primary cause of shock, and whether terminal shock may actually result from potassium poisoning caused by release of potassium from intracellular spaces has remained unanswered.[1-3] Furthermore, the theory that changes in the blood potassium level are not the cause of shock, but that such changes are incidental to alterations in blood volume has not been substantiated.

The present work was undertaken to ascertain what variations in the potassium level take place during shock caused by induced hyperpyrexia. This report includes experiments on patients who were

---

[1] Scudder, J., Zwemer, R. L., and Truszkowski, R., *Surgery,* 1937, **1**, 74.

[2] Scudder, J., Zwemer, R. L., and Whipple, A. O., *Ann. Surg.,* 1938, **107**, 161.

[3] Bisgard, J. D., McIntyre, A. R., and Osheroff, W., *Surgery,* 1938, **4**, 528.

undergoing treatment in the Burdick fever cabinet, and on others who were being treated by induced malaria shock.

In order to ascertain whether changes in the degree of hydration of the blood might affect the potassium level, a hematocrit determination was made on each sample of blood taken from the patient. Since variations in the concentration of one inorganic constituent of the blood might reasonably be expected to produce alterations in that of others, also, sodium and calcium determinations were made concurrently with the potassium and hematocrit studies. In addition, blood sugar estimations were made during progression of the induced shock.

Methods. The sodium content was determined by Salit's[4] triple acetate method, and that of potassium by the method of Truszkowski and Zwemer,[5] as modified by Harris.[6] The concentration of calcium was determined by the Clark-Collip modification of the Kramer-Tisdall method.[7] Blood sugar determinations were made by the micro method of Gibson.[8] Hematocrit readings were obtained by centrifuging 4-5 ml of fluorinated blood in a graduated centrifuge tube for 20 minutes, and rechecking after an additional 5 minutes of centrifugation.

In order to study the variations in blood serum potassium in induced hyperpyrexia, and to correlate such variations with changes in blood concentration, three determinations were made on each patient studied.

A. Fever Therapy. The first blood sample was drawn from each patient at the end of a 12-hour fast, and immediately before an intravenous injection of about 1500 ml of 5% glucose in physiologic saline. The intravenous injection was followed by induced hyperpyrexia in a Burdick fever therapy cabinet. At the height of the fever, which usually occurred about 4 to 5 hours after treatment was begun, a second sample was taken. After treatment was discontinued, and the patient's temperature had returned to normal, a third sample was taken.

All blood samples were withdrawn in amounts of approximately 30 ml from a forearm vein. About 4 ml of blood were immediately placed in a graduated, 15 ml centrifuge tube containing sufficient NaF to prevent clotting, and centrifuged at once for 20 minutes. A reading of total volume and cell volume was then taken, and the tube was then centrifuged an additional 5 minutes to check for variations.

---

[4] Salit, B. W., *J. Biol. Chem.*, 1932, **96**, 659.

[5] Truszkowski, R., and Zwemer, R. L., *Biochem. J.*, 1937, **30**, 1345.

[6] Harris, J. E., personal communication.

[7] Clark, E. P., and Collip, J. B., *J. Biol. Chem.*, 1925, **63**, 461.

[8] Gibson, R. B., PROC. SOC. EXP. BIOL. AND MED., 1930, **27**, 480.

The remaining portion of the sample was placed in a small bottle, and defibrinated by means of a paper clip. A 0.2 ml portion of this was used for a sugar determination, and the remainder was immediately centrifuged. The serum was pipetted off at once, and stored in the refrigerator until all three samples had been thus treated. When all 3 serum specimens were available, the protein-free filtrate was immediately obtained for the potassium determinations. Therefore, the amount of time during which the cells were in contact with the serum never exceeded 15 minutes.

During the course of the treatment, the patient received fluids, in the form of water and cracked ice, in an amount sufficient to control thirst and prevent excessive dehydration from loss of body fluids. Saline tablets were also administered occasionally. No food was taken until after the final blood sample was drawn.

B. Malaria Shock Therapy. Parallel studies were made on patients undergoing malarial shock treatment. In these cases the plan was to ascertain what variations in the blood serum occurred during the chill caused by the treatment. The patient's history was studied in the hope of anticipating the probable time of occurrence of the chill. A blood sample was drawn when there were indications of an impending chill, but before the temperature had risen above normal. The progress of the fever was followed, and, when it was at its height, a second sample was taken. A third sample was obtained after the temperature had returned to normal. The general procedure of handling the samples was the same as in the fever therapy cases.

Discussion. Our analyses showed that there was no uniform variation in the blood potassium level during the course of treatment, which indicates that an increase in the potassium content of the blood is not a factor in the shock caused by hyperpyrexia. Contrariwise, the results suggest that the blood potassium level may vary directly with the degree of hydration of the blood. No significant changes in Na or Ca concentration occurred as a result of fever treatment, and the blood sugar curves followed no regular pattern.

The hematocrit determinations showed that the degree of hydration of the blood was maintained within narrow limits during the fever therapy treatments. This was accomplished by the intravenous administration of fluids before treatment, together with the oral administration of water and saline tablets. There were no indications of severe shock.

Summary. In 15 cases of induced hyperpyrexia, there were no changes in the potassium content of the blood which would indicate

TABLE I.
Variations of Electrolytes of Blood Serum in Hyperthermia.

Case	Treatment No.	Temperature variations (rectal)		Hematocrit % cell volume			Blood Sugar Mg%			Potassium Mg%			Sodium Mg%			Calcium Mg%		
		(2)	(3)	(1)	(2)	(3)	(1)	(2)	(3)	(1)	(2)	(3)	(1)	(2)	(3)	(1)	(2)	(3)
1	3	105.6	100.4	x	41.5	33.3	114	109	109	16.7	22.6	22.6	314.7	319.8	319.8	10.7	10.7	10.6
2	10	102.5	100.0	41.0	36.1	34.9	91	100	80	18.2	22.5	22.2	321.2	349.7	342.1	9.7	10.2	10.2
3	1	105.8	99.4	31.8	35.5	40.0	157	120	122	22.0	20.4	18.9	351.3	348.2	348.2	10.0	10.0	9.9
4	9	105.8	101.0	36.3	37.1	36.27	78	94	126	18.2	17.9	19.1	314	322	325	11.5	11.9	11.1
3	3	105.8	99.2	33.3	32.4	34.22	147	107	90	21.1	20.4	20.8	322.5	322.5	322.5	9.7	10.5	10.3
1	6	105.6	100.8	33.3	33.3	33.3	x	98	81	18.5	19.8	20.4	319.9	322.5	322.5	10.4	10.9	11.5
3	7	104.5	99.6	32.9	31.0	31.0	133	112	109	19.2	20.2	20.8	322.5	325.1	317.3	12.0	11.2	11.7
5	1	103.4	100.0	41.8	46.8	45.3	116	96	85	16.3	17.5	16.7	327.9	353.8	334.5	9.2	10.7	10.7
6	*7	104.0	100.0	42.3	40.6	41.5	85	109	189	18.0	21.1	19.2	336.3	333.4	325.2	10.3	10.6	9.7
7	1	104.0	100.6	22.0	22.0	21.8	157	68	96	x	x	x	300.3	300.3	300.3	8.5	8.4	8.3
8	1	104.6	100.4	33.3	31.7	32.4	165	100	110	x	x	x	348.1	348.1	339.2	9.0	9.2	9.2
8	3	104.4	100.0	28.9	29.3	34.6	131	84	100	18.0	18.0	17.4	—	—	—	—	—	—
7	3	104.0	100.8	12.2	16.3	14.3	—	—	—	21.3	22.2	20.8	—	—	—	—	—	—
9		105.8	100.0	36.4	36.4	39.3	100	129	105	21.7	21.3	20.0	345.1	345.1	342.1	9.7	9.7	9.8
10		104.8	100.0	29.0	29.1	28.0	120	104	94	19.3	19.0	20.3	x	314.7	322.5	10.3	10.3	9.9

*No intravenous injection preceding treatment. Final sample taken 20 minutes following an intravenous injection of 1000 ml of 5% glucose in saline (physiologic).

(1) Normal conditions before treatment; (2) Height of fever; (3) Return to normal.

— Determination not made.

x Duplicate values not satisfactory.

Patients, 1-8, Burdick fever cabinet therapy.

Patients 9-10, Malaria shock therapy.

that an increase in the amount of potassium is a factor in shock. The blood potassium level tends to vary directly with the degree of hydration of the blood. There were no significant changes in the Na, Ca, or blood sugar levels, produced by this condition.

11706

Toxic Substance in Extracts of Postpartum Rabbit Uterus.*

L. Sapirstein, W. Pollock and B. Krichesky.
(Introduced by B. M. Allen.)

From the Department of Zoology, University of California at Los Angeles.

In a previous communication it was demonstrated that saline extracts of postpartum rabbit uterus were highly toxic after intravenous injection in the same species.[1] Death occurred with the characteristic symptoms of histamine shock and the activity of the extracts was destroyed by the enzyme histaminase.[2] It was tentatively concluded that the active material was histamine or a histamine-like substance.

In order to determine whether the effects of this "histamine-like substance" on the guinea pig ileum were identical with those of histamine, uterine extracts were subjected to acid hydrolysis, the hydrolysates neutralized and assayed on an isolated strip of guinea pig ileum, according to the method of Barsoum and Gaddum, and the responses compared to those of histamine.[3]

Extracts so treated assayed between 2.2 and 3.2 γ histamine per cc. This is approximately the histamine content of normal rabbit tissues, and would be by no means sufficient to cause death in the amounts injected.[4] It was possible, moreover, to recover between 70 and 80% of histamine dichloride added to saline extracts of rabbit uterus and of various guinea pig tissues in quantities between 10 and 60 γ per cc and treated in the same manner (Table I). The failure to recover toxic quantities of histamine from uterine extracts led to the conclusion that some substance other than histamine is responsible for the toxic activity of the extracts.

It was considered possible that the active substance was protein-

---

* Aided by a research grant from the University of California.

[1] Krichesky, B., and Pollock, W., *Science*, 1940, **91**, 410.

[2] Krichesky, B., and Pollock, W., *Am. J. Physiol.*, 1940, in press.

[3] Barsoum, G. E., and Gaddum, J. H., *J. Physiol.*, 1935, **85**, 1.

[4] Best, C. H., and McHenry, E. A., *Physiol. Rev.*, 1931, **11**, 371.

TABLE I.
Assay of Histamine Containing Tissue Extracts.

Solution assayed	Amt histamine added per cc, γ	Amt histamine† per cc (assay) γ	% Recovery*
Guinea pig extract	25	19	76
Same	25	19.5	78
Guinea pig heart and kidney extract	16.5	12.0	72
Same	16.5	12.5	75
Postpartum rabbit uterus extract, 1.0 cc toxic	25	19	76
Postpartum uterine extract, 1.0 cc toxic	10	9.2	92
Same	60	50	83
Same	0.0	2.5	—
Same but 1.6 cc toxic	0.0	2.2	—
Same	0.0	3.2	—

†Calculated by comparison of height of contraction with histamine standards. Doses assayed were usually equivalent to about .2 γ of histamine.

*These percentage recoveries are given in terms of added histamine. The recovery figures for rabbit uterus extract which contains small amounts of native histamine are consequently somewhat too high.

like in nature. Filtrates of 4 toxic extracts through a medium Berkefeld filter were no longer active. Treatment of 2 toxic extracts with N and N/20 NaOH in the cold resulted in complete loss of toxicity. N HC1 was found to have the same effect. Heating at 80° C for 5 minutes in order to denature the proteins in the extract was found to reduce the toxicity of the extract 2 to 5 times, but failed to destroy it. These heated extracts, however, contained abundant precipitates of protein which may have caused death by embolism. It was impossible to bring this precipitated material into solution by changing the pH as both acid and alkali had been shown (vide supra) to destroy the activity. Two extracts incubated for 24 hours with pancreatin were also toxic though in doses 5 and 6 times as large as untreated extracts. However, a control solution incubated with a pancreatin solution which had been boiled previously to destroy its enzyme activity was also found to be one fifth as toxic as untreated extracts. Consequently, the loss of toxicity cannot be attributed to enzyme action.

Summary and Conclusions. The reactions of the guinea pig ileum to the toxic substance indicate that the latter is not histamine. It is, however, inactivated by histaminase suggesting that histaminase is not specific for histamine but may attack other substances.[2] Zeller has shown that histaminase is capable of hydrolyzing several diamine compounds other than histamine.[5]

The toxic substance is destroyed by mild treatment with acid or alkali. However, heating at temperatures sufficient to denature proteins present failed to effect complete destruction of the toxicity. Treatment of the extract with both boiled and unboiled solutions of pancreatin discloses no destruction of the toxic substance by enzyme action.

---

[5] Zellers, E. A., *Helvetia Chimica Acta*, 1939, **22**, 1381.

11707

Modified Method for Determination of Certain Organic Iodine Compounds, Inorganic Iodide in Plasma and Urine.*

H. L. WHITE AND DORIS ROLF.

From the Department of Physiology, Washington University School of Medicine, St. Louis.

A method for the determination of diodrast and inorganic iodide iodine in blood and urine was presented previously.[1] The principles involved were acid permanganate digestion of the sample with oxidation of iodine to iodate, nitrite reduction of the permanganate, destruction of excess nitrite with urea, and titration of the iodate with thiosulphate in an excess of potassium iodide. This procedure could be carried out either by hand heating of the individual tubes over a micro burner or by heating a number of tubes in a boiling water bath. The digestion of individual samples by hand yields accurate results but requires the constant attention of the analyst. The water bath digestion, however, involves some error, particularly in the digestion of plasma filtrates at high iodine levels, and it is sometimes more difficult to remove all of the permanganate because some manganese dioxide adheres firmly to the walls of the tubes. It is shown here that with an alkaline permanganate digestion these difficulties with the water bath heating are eliminated.

With an alkaline digestion more permanganate must be used, and a longer heating period allowed than with the acid digestion. In order to keep down the volume of the digest at time of nitrite treatment and at subsequent titration, preliminary evaporation of filtrate samples is carried out.

Procedure. Reagents: 13% trichloracetic acid (A.R.), 72% sodium hydroxide (A.R.), approximately 0.4 M potassium permanganate, 4 N sulfuric acid, 1.0 M sodium nitrite, 5 M urea, granulated potassium iodide, 1% starch, 0.0005 N sodium thiosulfate or 0.0025 N sodium thiosulfate.

---

* Aided by a grant from the Commonwealth Fund.

[1] White, H. L., and Rolf, D., PROC. SOC. EXP. BIOL. AND MED., 1940, **43**, 1.

Apparatus: circular test-tube rack, water bath, 18x150 mm test tubes, dropping bottles, burette calibrated to 0.01 cc or 0.05 cc.

In preparing the potassium permanganate make a 7% solution, boil for 5 to 10 minutes and let stand for at least 24 hours. After standing, filter through asbestos or sintered glass and standardize. This standardization need not be extremely accurate, since it is only necessary to establish that the permanganate is at least 0.4 M. If it is more than 0.4 M, use the quantities given in the procedure but if it is less than 0.4 M add a correspondingly greater amount.

PLASMA. Precipitation. Pipette 1 volume of plasma into a small Erlenmeyer flask, add 7 volumes of water and 2 of 13% trichloracetic acid, shake and filter after about 5 minutes.

Digestion. Pipette 3 cc of filtrate into an 18x150 mm test tube, place the tube in a rack and evaporate the filtrate to a volume of about 1.5 cc by suspending the rack in a boiling water bath for 30 minutes. If 2 cc, or 4 or 5 cc, of filtrate are used, evaporate 10 minutes and 40 minutes, respectively. If 1 cc of filtrate is used add 0.5 cc of water and do not evaporate. With an iodine level between 1 and 4 mg%, 3 cc of filtrate will be sufficient; below 1 mg%, 4 or 5 cc is advisable. Above 5 mg%, 2 cc or 1 cc may be used, and if the level is above 20 mg% it is necessary to dilute the filtrate. After evaporation remove the rack from the bath, add 0.1 cc of 72% sodium hydroxide, 1 cc of 0.4 M permanganate, shake the individual tubes gently, place the rack in the boiling water bath for 15 minutes and shake the tubes every 4 to 5 minutes. The tubes can be easily shaken if one uses a piece of gauze to protect the hands. Gummed labels identify the samples. After 15 minutes remove the rack from the bath, add 0.8 cc 4 N sulfuric acid and mix by shaking. Treating each tube individually, add 1 M sodium nitrite from a dropping bottle until the permanganate is almost gone; finish the treatment with 0.5 M sodium nitrite, adding one full drop in excess. Shake the tubes after the addition of each drop of nitrite; wash the walls of the tubes by tilting them so that all traces of permanganate are removed. Place the rack in the bath for 2 minutes; during this interval frequently lift each tube from the bath and shake. At the end of 2 minutes remove the rack from the bath, add 4 drops of 5 M urea from a dropping bottle so that the drops wash down the walls of the tubes. Replace the rack in the bath for 1 minute. After about 30 seconds, lift each tube from the rack and shake. At the end of the minute, remove the rack from the bath and wash the walls of the tubes with the hot digest, tilting the tubes so that the solution reaches any portion which may have been touched by nitrite. Place the rack in the bath for about 1 minute longer, repeating the procedure outlined in the preceding two sentences. One can easily handle 6 to 8 samples at one time but it is not advisable to treat more than 10. The digestion and subsequent treatment of 10 tubes after evaporation has been completed will take about 25 to 30 minutes.

Titration. Titrate under artificial light with 0.0005 N thiosulphate, in an excess (few tenths of a milligram) of potassium iodide.† No measurable blank is obtained on water. A blank titration on 3 cc of normal plasma filtrate will be from 0.003 to 0.01 cc; when this is disregarded practically 100% recoveries are obtained, which indicates that a very slight loss of iodine is thereby compensated. Recoveries of the iodine of diodrast added to plasma filtrate are shown in Table I.

URINE. Digestion. Dilute the urine so that 1 cc contains from 5 to 500 μg of iodine. Place 1 cc of diluted urine plus about 0.5 cc of water in a test tube and treat exactly as outlined for the filtrates except that 0.6 cc of permanganate is used.

Titration. If the sample contains 60 μg or more of iodine, titration can be carried out in the daylight, using a burette calibrated to 0.05 cc and 0.0025 N thiosulfate. Employing this titration the tubes need only to be cooled to room temperature. More potassium iodide (1

TABLE I.

Percentage Recovery of Diodrast Added to Normal Plasma Filtrate. A 1 mg % filtrate means that 1 mg of diodrast iodine has been added to 1000 cc of a 1 to 10 filtrate of normal plasma.

1 mg % filtrate			2 mg % filtrate	4 mg % filtrate		10 mg % filtrate	
3 cc	4 cc	5 cc	3 cc	2 cc	3 cc	1 cc	2 cc
101.0	99.3	100.2	99.3	98.6	100.5	99.4	97.6
95.8	98.7	105.8	99.1	98.6	98.4	99.1	99.2
102.0		102.5	99.8	99.8	99.8	99.4	99.6
99.0		97.5	100.5	98.2	100.6	100.0	99.0
102.7		97.5	102.2	97.6	99.1	100.0	
97.3			99.0	97.5	98.1		
			97.3	97.6	98.5		
			99.4	98.8			
				98.5			
				97.5			

---

† It has come to our attention that the direction in our original communication to add "a few crystals" of potassium iodide has sometimes been misinterpreted. We use a few granules of "granulated" potassium iodide, totaling about 0.5 mg. If one takes a few crystals of "crystalline" potassium iodide he will have many times the optimum amount, with the result that significant amounts of iodine will be liberated, leading to an erroneously high titration and inability to reach a definite endpoint. Furthermore, the direction to "titrate in an artificially lighted room" means that the room should be reasonably dark in the absence of the artificial light.

TABLE II.
Percentage Recovery of Iodine from Aqueous Solutions.

	Amount of iodine in sample						
	5 μg	7.5 μg	10 μg	100 μg	200 μg	300 μg	500 μg
Aqueous solution of diodrast	99.9	99.9	98.7	101.0	99.4	99.4	99.6
	101.9	100.0	100.4	100.0	99.3	100.2	100.5
	99.2	98.9	100.0	100.0	99.3	99.4	100.6
	100.0	99.9	99.4				100.3
	101.0	101.0	99.4				
	100.0	99.6	99.5				
	100.4		99.2				
	99.4		99.9				
	99.8		100.0				
	99.4		99.5				
	99.8						
Aqueous solution of diodrast compound				100.5			
				100.2			
				100.5			
Aqueous solution of KI				101.5			
				100.5			
				101.5			

to 10 mg) is needed with 50 to 500 μg iodine in the sample than is needed for the "micro" titration. If the sample contains less than 25 μg of iodine, the titration should be carried out under artificial light at 10° C or lower, using 0.0005 N thiosulphate and a burette graduated to 0.01 cc.[1]

This same procedure has been used for the determination of water solutions of potassium iodide and diodrast compound, recovery being complete (See Table II). A few determinations on water solutions of hippuran have yielded 97 to 98% recovery when the alkaline digestion is carried on for 75 minutes. In this case it is necessary to use 1.2 cc of 0.4 M permanganate with periodic addition of distilled water to keep the volume up to 1.5 to 2 cc, and to add 1 cc of 4 N sulfuric acid before the addition of sodium nitrite. Some cloudiness is observed in hippuran digests so treated; this no doubt somewhat increases the error of the titration but has not prevented recoveries of the accuracy mentioned.

It was pointed out before[1] that some diodrast is carried down during the plasma precipitation, but that the percentage recovery from the filtrate of known amounts of diodrast added to the plasma before precipitation is constant on separate precipitations of the same plasma, even at various iodine levels (2.5 to 30 mg I per 100 cc). It was further shown that this percentage recovery does not vary greatly with different normal subjects. The previous average figures for the percentage of diodrast coming through in plasma filtrates of normal humans and dogs were 84.6 and 84.7%, respectively. More recent findings place these averages at 86.9% for humans and 85.9% for dogs, with average deviations of 1.7% and 1.4% respectively. This figure can be established for each subject when the highest accuracy is desired.

CALCULATIONS. For plasma filtrate,

$$\text{cc } 0.0005 \text{ N thiosulphate} \times \frac{10.6}{1000} \times \text{dilution factor} \times \frac{100}{\%\text{ coming through}}$$
$$= \text{mg iodine per 100 cc plasma.}$$

Example, using 3 cc of plasma filtrate,

$$0.530 \times \frac{10.6}{1000} \times \frac{1000}{3} \times \frac{100}{86.9} = 2.15 \text{ mg iodine per 100 cc plasma.}$$

For urine,

(1) using 0.0005 N thiosulphate,

$$\text{cc thiosulphate} \times \frac{10.6}{1000} \times \text{dilution factor} \times 100 = \text{mg iodine per 100 cc urine.}$$

(2) using 0.0025 N thiosulphate,

$$\text{cc thiosulphate} \times \frac{53}{1000} \times \text{dilution factor} \times 100 = \text{mg iodine per 100 cc urine.}$$

11708

Degree of Ketosis During Fasting.

ARNE N. WICK, JAMES W. SHERRILL AND EATON M. MACKAY.
From the Scripps Metabolic Clinic, La Jolla, California.

Ketosis during extended fasting has heretofore been measured by the ketonuria. Because the acetone bodies are threshold substances the ketonuria does not necessarily give a true picture of the degree of ketosis which exists. Furthermore the only record of even the ketonuria in fasts of more than 3 or 4 days' duration were obtained prior to the development of modern methods for determining acetone bodies. We have examined the degree of ketosis during fasting in a human subject and in a group of rats through measurements twice daily of the level of acetone bodies in the blood. In the rats arterial blood and in the human subject venous blood was used for this purpose. Due to the utilization of acetone bodies by the tissues during a ketosis[1] the figures for acetone bodies for venous blood would be slightly lower than for arterial blood. The method of Barnes and Wick[2] was used for the determination of acetone bodies. Forty adult female rats weighing an average of 175 g each were used for the rat experiment, being fasted from the stock diet.[3] Two rats were sacrificed every 12 hours and the averages of the results obtained on these pairs of samples are reported here. The human subject was a female 52 years of age, 177 cm in height and weighing 121 kg. Except for the marked obesity she was in no way abnormal. She had been taking a general diet prior to fasting.

Our results are charted in Fig. 1. During a period of 48 hours in the rat and 85 hours in the human subject after the beginning of fasting the concentration of acetone bodies in the blood rose to reach a level which was then maintained with some fluctuation until the end of the 10-day fast. The variability of the blood acetone body level during fasting appears to be greater in the rat but it must be remembered that the rat data was obtained from a group while the human data was obtained from a single subject. The rate of increase in the degree of ketosis at the onset of fasting is probably determined by the extent of the stored carbohydrate and protein readily available for catabolism during this period.[3, 4] The main-

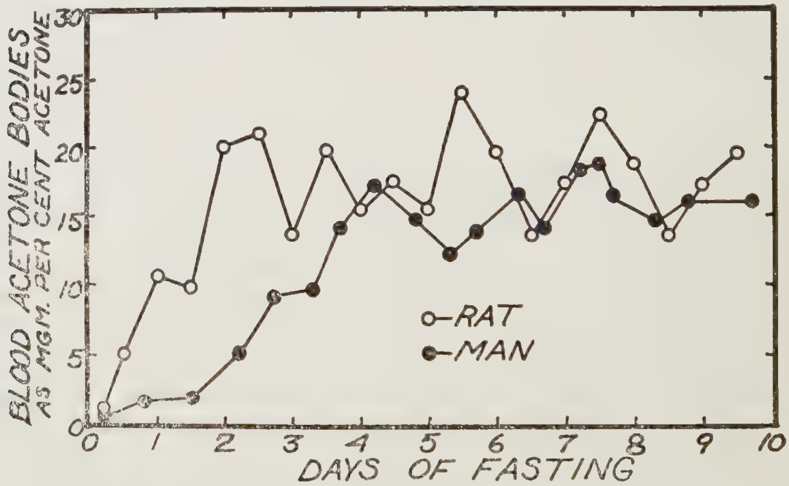


FIG. 1.

Blood Acetone Body Concentration During Fasting in Man and the Rat.

---

[1] Barnes, R. H., Drury, D. R., Greeley, P. O., and Wick, A. N., *Am. J. Physiol.*, 1940, **130**, 144.

[2] Barnes, R. H., and Wick, A. N., *J. Biol. Chem.*, 1939, **131**, 413.

[3] Wick, A. N., and MacKay, E. M., *Am. J. Physiol.*, 1940, **130**, 332.

[4] MacKay, E. M., Carnes, H. O., and Wick, A. N., PROC. SOC. EXP. BIOL. AND MED., 1939, **41**, 40.

tenance of the ketosis at a more or less constant level after this period is probably due to the availability for catabolism of a limited but relatively constant source of carbohydrate from tissue protein and the glycerol from the fat which is burned.

Summary. During the first 2-4 days of fasting the degree of ketosis in the human and the rat as measured by the blood acetone body concentration gradually increased to a level of 15-20 mg % (acetone) which then tended to be maintained during the rest of the 10-day fasting period.

11709

Development of Influenzal Complement-Fixing Antigen and Antibody in Mice.*

Monroe D. Eaton, M. D., and C. R. Nicewonger

From the Research Laboratory of the California State Department of Public Health, Berkeley, California.

The lungs of mice infected with the virus of epidemic influenza contain a soluble complement-fixing antigen which is separable from the virus.[1, 2] The purpose of the work here reported was to investigate the conditions under which this antigen and its corresponding antibody are formed.

Mice were inoculated with the mouse passage strain PR8 of epidemic influenza virus in dilutions from 10^{-2} to 10^{-8} of lung. At different intervals of time from 1 to 10 days, 6 to 8 mice receiving each of the dilutions were killed by bleeding from the heart under chloroform anesthesia. The complement-fixing antigen was measured by titration of a saline suspension of the ground lung material against a constant dilution of 1:20 of a pool of human convalescent serum according to the complement-fixation method previously described.[3]

The results are recorded in the accompanying table. One day after inoculation none of the mice showed macroscopically visible

---

* These studies were made under the auspices, and with the support, of the International Health Division of The Rockefeller Foundation cooperating with the California State Department of Public Health.

[1] Smith, W., *Lancet*, 1936, **2**, 1256.

[2] Hoyle, L., and Fairbrother, R. W., *J. Hygiene*, 1937, **37**, 512.

[3] Eaton, M. D., and Rickard, E. R., *Am. J. Hygiene*, 1940, in press.

TABLE I.

Lesions and Complement-Fixing Antigen in Lungs of Mice at Various Times After Inoculation with the PR8 Strain of Epidemic Influenza Virus.

Day killed	Gross lung lesions: % tissue involved*	†Titer of complement-fixing antigen in lungs of mice inoculated with dilution of			
		10^{-3}	10^{-4}	10^{-5}	10^{-6}
1	0	480	320	240	80
2	20-3	640	640	480	240
3	30-6	1280	960	480	480
4	50-20	640	960	640	480
5	70-20	—	640	640	480
6	90-30	—	—	640	480
7-8	70-50	—	—	40	40
9-10	50	—	—	—	less than 20

*This column shows the range of lung lesions in living mice which had received the dilutions 10^{-3} to 10^{-6}, the degree of lung involvement depending upon the amount of virus inoculated.

†Titer given as dilution of whole wet mouse lung.

— Titration not done because mice had died.

lung lesions, but the complement-fixing antigen was already present in relatively high titer except in the group receiving a dilution of 10^{-6}. Between the second and fifth days the appearance of antigen at a maximum titer preceded by 2 to 3 days the maximum development of lung lesions. This suggests that the formation of the antigen is associated with a rapid multiplication of the virus before the appearance of the red pulmonary consolidation. With virus dilutions of 10^{-3} or above, the maximum titer of antigen seemed to be related to the amount of virus inoculated. In the lungs of mice inoculated with dilutions of 10^{-7} and 10^{-8} and killed after 4 days, the antigen titered 1:40 and 1:20 respectively and no macroscopic lesions were found.

At no time during the course of the infection was influenzal antigen detected in the serum of the mice receiving the various dilutions of virus.

The serum of mice inoculated with a dilution of 10^{-5} and bled 6 days later showed barely detectable neutralizing antibodies against 10 M.L.D. of virus. At 7 to 8 days the serum neutralized 10 to 100 M.L.D. of virus. The disappearance of antigen from the lungs at 7 to 8 days corresponded closely to the appearance of neutralizing antibodies in the serum. Virus was detectable in the lungs of mice by subinoculation for as long as 21 days, but the titer diminished progressively after 7 or 8 days.

Even though the serum of mice inoculated with a dilution of 10^{-6} showed strong neutralizing properties at 10 days, no complement-fixing antibodies were detectable until 14 days after inoculation, and then only at serum dilutions of 1:2 to 1:4. Mice receiving 1/100 to 1/1,000 M.L.D. of virus (dilutions of 10^{-7} and 10^{-8}) failed to develop complement-fixing antibodies, although some neutralizing antibodies were detectable. One intraperitoneal inoculation with 100,000 intranasal M.L.D. of virus caused the production of only small amounts of complement-fixing antibodies.

In contrast to results following one inoculation, a second or third inoculation by the intranasal or intraperitoneal routes stimulated the production of complement-fixing antibodies up to titers of 1:64 to 1:128. This marked secondary response occurred in actively immune mice where there was little multiplication of virus or formation of complement-fixing antigen.

In human beings, except the very young, the formation of influenzal complement-fixing antibodies following infection is probably a secondary immune response conditioned by previous exposure to the virus. The results with mice suggest that after the first infection with small amounts of the virus of epidemic influenza, complement-fixing antibodies may not appear in the serum in significant titer.

11710

Whole Blood and Plasma Ascorbic Acid Concentrations in Patients with Pellagra and Associated Deficiency Diseases.*

Virginia Minnich, Sydney T. Wright, Carl V. Moore, and Tom D. Spies.

From the Department of Internal Medicine, Washington University School of Medicine, and Department of Internal Medicine, University of Cincinnati.

The great majority of patients who attend the Nutrition Clinic of the Hillman Hospital have existed for most of their lives on grossly inadequate diets. Practically all have, or have had, clinical pellagra, beriberi, ariboflavinosis, or other deficiency diseases. Many have clinically recognizable manifestations of several deficiency states. An occasional patient is found to have spongy, bleeding gums which suggest an associated hypovitaminosis C, but advanced cases of scurvy are infrequently seen despite the fact that foods rich in ascorbic acid are available to these people only during the late spring and summer.[1] The small quantity of fruits they can afford to preserve is exhausted late in the fall so that for most of the year they have an extremely limited intake of the anti-scorbutic vitamin. In order to determine the degree of their vitamin C depletion, whole blood and plasma ascorbic acid concentrations were measured on 70 of these persons. These observations form the basis of the present report.

---

* University of Cincinnati Studies in Nutrition at the Hillman Hospital, Birmingham, Alabama. These studies were aided by a grant from Mead Johnson and Company.

Recent experimentation has shown that whole blood or blood cell concentrations of ascorbic acid provide, in all probability, as reliable an index of the adequacy of vitamin C reserves as is available.[2–5] Determination of the amount of ascorbic acid in plasma alone, on the other hand, has been shown to be of very limited value. This fraction is quite labile and changes rapidly with the addition or withdrawal of vitamin C from the diet.[5–8] The ascorbic acid content of the cellular elements of the blood, however, is much more stable.[5, 8] It has been observed that as a state of vitamin C unsaturation develops, ascorbic acid is first depleted from the plasma, then from the red cells, and lastly from the platelet and white cell elements. In the investigation reported by Crandon and Lund[8] in which a normal male subject voluntarily placed himself on a vitamin C free diet, the plasma was free of ascorbic acid within 11 days, but 4 months were required to deplete the white cell-platelet fraction. Conversely, it has been determined that when ascorbic acid is given to a person with scurvy, vitamin C (postabsorptive) first appears in the leucocytes, next in the red cells, and lastly in the plasma.[5] Consequently, a high fasting level of ascorbic acid in plasma provides a good indication that vitamin C stores are adequate while decreased concentrations in the cellular elements of blood serve as an index of vitamin C deficiency or unsaturation. Low levels of plasma ascorbic acid do not necessarily imply a significant depletion of the vitamin.

The methods of Butler and Cushman[5] and of Mindlin and Butler[9] were used for the determination of whole blood and of plasma ascorbic acid concentrations in 70 patients from the Clinic group and in 20 "normal" adult laboratory workers. No attempt was made to measure separately the apparent ascorbic acid content of leucocytes. For the whole blood determinations, filtrates were used from blood which had been saturated with CO before precipitation with metaphosphoric acid.[5] Determinations, except for a few on the control subjects, were made between June 15 and July 1.

---

[1] Spies, T. D., Swain, A. P., and Grant, J. M., *Am. J. Med. Sc.*, 1940, **200**, 536.

[2] Eekelen, M. von, Emmerie, A., and Wolff, L. K., *Z. Physiol. Vitaminforsch.*, 1937, **6**, 150.

[3] Nevweiler, W., *Z. Physiol. Vitaminforsch.*, 1938, **7**, 128.

[4] Heinemann, M., *J. Clin. Invest.*, 1937, **17**, 751.

[5] Butler, A. M., and Cushman, M., *J. Clin. Invest.*, 1940, **19**, 459.

[6] Greenberg, L. D., Rinehart, J. F., and Phatak, N. M., PROC. SOC. EXP. BIOL. AND MED., 1936, **35**, 135.

[7] Mindlin, R. L., *J. Pediat.*, 1940, **16**, 275.

[8] Crandon, J. H., and Lund, C. C., *New England J. Med.*, 1940, **222**, 748.

[9] Mindlin, R. L., and Butler, A. M., *J. Biol. Chem.*, 1938, **122**, 673.

Whole Blood Ascorbic Acid. The data is summarized in Table I. Patients were classified according to their primary clinical manifestations. Whole blood ascorbic acid concentrations in the control subjects varied from 0.95 mg % to 1.46 mg %. This range of normal values agrees well with those recorded in the literature.[2–5] The average for the clinic group, however, was much lower (0.63 mg % as compared with 1.20 mg %). Eight (11%) of the patients had values less than 0.3 mg % and 51 (73%) had values less than 0.8 mg %. This distribution analysis is expressed in Fig. 1.

Plasma Ascorbic Acid. Plasma ascorbic acid concentrations were, as one would expect, invariably lower than those for whole

TABLE I.
Ascorbic Acid Concentration of Whole Blood and of Plasma in Patients with Deficiency Disease.

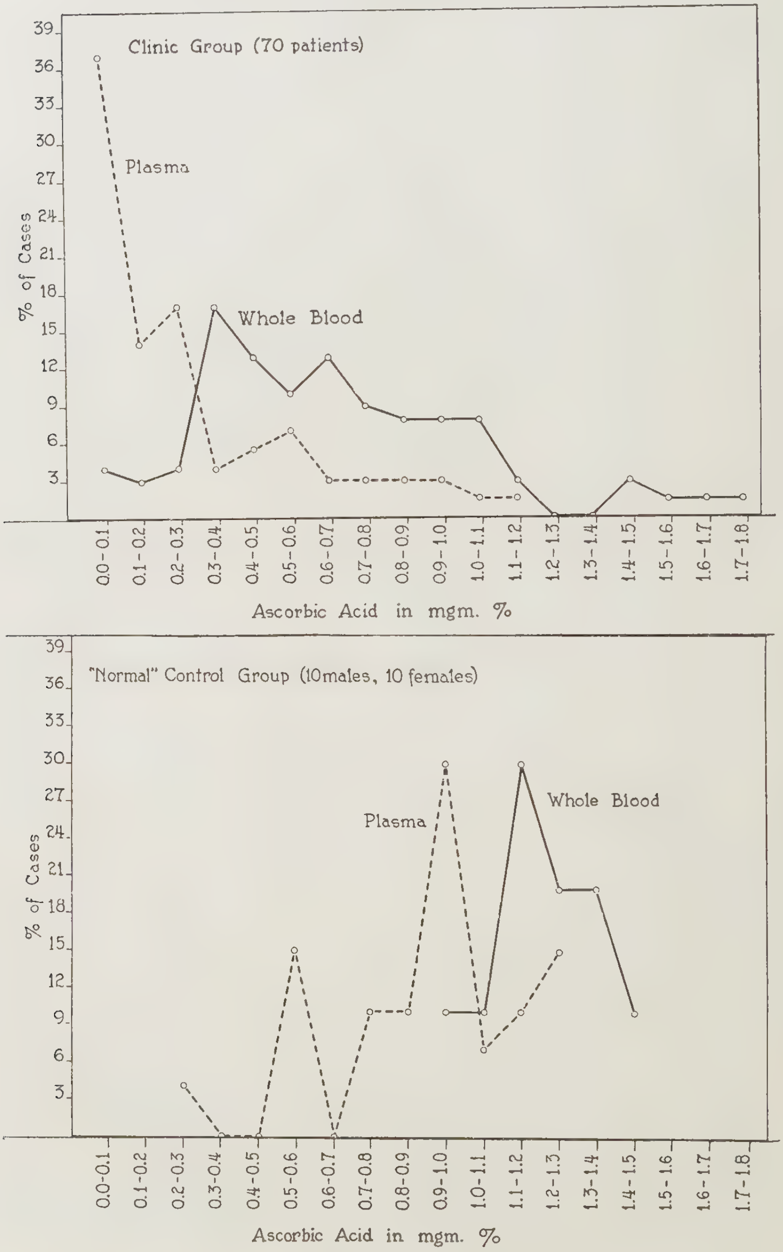
		Whole blood ascorbic acid			Plasma ascorbic acid		
Diagnosis*	No. of cases	Avg mg%	Min. mg%	Max. mg%	Avg mg%	Min. mg%	Max. mg%
Thiamin deficiency	4	0.61	0.46	0.76	0.21	0.0	0.35
Riboflavin deficiency	13	0.74	0.33	1.49	0.33	0.0	0.78
Nicotinic acid deficiency	14	0.47	0.17	0.96	0.15	0.0	0.59
Subclinical nutritional deficiency states†	23	0.79	0.28	1.77	0.32	0.0	1.12
Purpura (nutritional?)	5	0.60	0.07	1.00	0.42	0.0	0.94
Anemias							
Macrocytic	7	0.22	0.0	0.74	0.09	0.0	0.42
Hypochromic, microcytic	4	0.72	0.26	1.52	0.40	0.0	1.02
Summary of all patients studied	70	0.63	0.0	1.77	0.27	0.0	1.12
Normal adult subjects							
Men	10	1.14	0.95	1.46	0.74	0.22	1.10
Women	10	1.27	1.12	1.46	1.03	0.78	1.25

*Many of these patients had clinical manifestations of more than one deficiency disease. To simplify the tabulation, they were classified under the most prominent of their several deficiencies.

†Patients who had been on inadequate diets and who complained of anorexia, burning of the tongue and "in the stomach," burning of the skin over the face. neck, and arms, paraesthesias of the hands and feet, nervousness, irritability, etc. Nearly all the members of this group had had pellagra in previous years.

Fig. 1

Distribution of Plasma and of Whole Blood Concentrations of Ascorbic Acid



blood. One of the "normal" subjects, a healthy male, had only 0.2 mg % ascorbic acid in his plasma; 3 other members of the control group had between 0.5 and 0.6 mg %; the other 16 had more than 0.8 mg %. For the patient group, there were 26 (37%) with less than 0.1 mg % plasma ascorbic acid, 48 (69%) with less than 0.3 mg %, 55 (79%) with less than 0.5 mg % and 64 (91%) with less than 0.8 mg %. (Fig. 1).

Since the diets of these patients provided them with only very inadequate amounts of vitamin C, the low values of plasma ascorbic acid were to be expected. It is of interest that even though the average concentration in whole blood was only half that of the control series, and even though 55 of the total number of 70 patients had whole blood ascorbic acid levels below the lowest value recorded for a normal subject, that there were only 8 patients lower than 0.3 mg %.† That is, if the amount of ascorbic acid in whole blood really constitutes an index of the adequacy of vitamin C nutrition, as seems to be the case, only 15 of the patients studied had normal vitamin C reserves, but in only 8 were the levels low enough to suggest that the depletion was advanced. The 4 normal subjects with low concentrations of plasma ascorbic acid were males; there was no significant variation in their whole blood ascorbic acid values from the rest of the members of the control group. From Table I it is obvious that there was no recognizable difference in the vitamin C blood levels of the patients with B_1 deficiency, ariboflavinosis, pellagra, etc. The low average for the macrocytic anemia group was caused by the low concentrations found in several patients critically ill with pernicious anemia, each of whom had approximately one million red cells.

Five of the 12 children studied had spongy, bleeding gums suggestive of a scorbutic change. Their whole blood vitamin C concentration, however, averaged no lower than that for the group as a whole (0.58 mg % with variation from 0.44 mg % to 0.98 mg %). Plasma ascorbic acid values for these 5 children ranged from 0.06 mg % to 0.71 mg %, averaged 0.31 mg %.

It is significant that these determinations were made during the last half of June. At that time tomatoes had not yet ripened. Wild blackberries had become edible only during the last few days of June. But fresh vegetables had been available to most patients for a month and many had had strawberries for 2 weeks. It is probable, therefore, that the blood levels of ascorbic acid were even lower before May 1st.

---

† This figure was arbitrarily chosen because it represented a fairly sharp dividing level, in terms of frequency, between values below and those above it.

The evidence accumulated in this investigation that patients with clinical deficiencies of the vitamin B complex frequently have an associated partial depletion of vitamin C is in agreement with the results reported by Goldsmith, Ogaard, and Gowe.[10] These workers studied the vitamin C nutrition of 18 pellagrins, and on 14 of these performed either oral or intravenous tolerance tests. Eight of their patients showed evidence of an ascorbic acid deficiency. Whole blood determinations of the vitamin were not done.

Summary and Conclusions. Whole blood and plasma ascorbic acid determinations made on 70 patients in the nutrition clinic of the Hillman Hospital indicate that 55 of these persons had a whole blood concentration of ascorbic acid lower than in normal controls. In 8 of the 70 cases, the concentration was so low as to suggest that depletion may have been advanced. Most of these patients had clinical evidence of other deficiencies, such as pellagra, beriberi and riboflavin deficiency, but no significant correlation could be made between the blood ascorbic acid values and the symptoms of these deficiency states, nor indeed of scurvy itself. These observations seem pertinent in view of the fact that they were made in late June, at a period when leafy vegetables and berries had been available to the patients for some time, and probably indicate that ascorbic acid deficiency is still greater at other periods. These studies give strong support to the concept that natural-occurring deficiency diseases exist as complexities rather than as single entities.

---

[10] Goldsmith, J. A., Ogaard, A. T., and Gowe, D. F., *Am. J. Med. Sc.*, 1940, **200**, 244.

11711 P

Mechanism of Liver and Kidney Damage Caused by Sodium Sulfamethylthiazole.*

DAVID LEHR, JACOB CHURG AND WILLIAM ANTOPOL.

From the Laboratories of the Newark Beth Israel Hospital, Newark, N. J.

In a previous investigation the occurrence of severe degenerative lesions in liver and kidney following the administration of sodium sulfamethylthiazole was observed. It was noted that histologically, the liver showed fatty degeneration and was almost devoid of glycogen, while the livers of rats receiving equimolar amounts of sulfapyridine and sulfathiazole were strikingly rich in glycogen. Increase in liver glycogen after the administration of sulfanilamide and sulfapyridine has been reported in the literature.[1, 2]

In order to analyze the different response to the administration of these 3 sulfanilamide derivatives the content of glycogen and drugs in the liver and the concentrations of the drugs in the blood were determined in experiments with rats. Urinalysis and determination of blood urea were used to indicate the functional state of the kidneys.

Results. Table I gives the results of a representative experiment.

The presence of high normal or increased amounts of glycogen in the liver after administration of sulfapyridine and sulfathiazole, as seen in histological sections, was confirmed by chemical analysis, as was also the glycogen depletion after sulfamethylthiazole.

The data seem to indicate that damage to the excretory function of the kidneys plays an important part in the production of the lesions after sulfamethylthiazole administration. In Table II the renal lesions caused by a single injection of sodium sulfamethylthiazole are clearly reflected in the urinary findings. Further proof of pronounced impairment of kidney function can be deduced from the high blood urea levels and retention of sulfamethylthiazole in the body.

The diminution or complete disappearance of acetylation in animals having toxic drug concentrations (Table I) can be regarded

---

* Aided by a grant from the Sidney C. Keller Research Fund.

[1] Greisheimer, E. M., Hafkesbring, R., and Magalhaes, H., *Proc. Am. Physiol. Soc.*, 52d Annual Meeting, 1940, p. 75.

[2] Antopol, W., and Robinson, H., *Arch. Path.*, 1940, **29**, 67.

TABLE I.

Groups of 5 rats received 3 intraperitoneal injections each, on 3 successive days, of 0.5 g/kg sodium sulfapyridine and equimolar amounts of the sodium salts of sulfathiazole (0.486 g/kg) and sulfamethylthiazole (0.515 g/kg), respectively. The controls were injected with equimolar amounts of sodium acetate (0.225 g/kg) in equal amounts of fluid. The animals were sacrificed 24 hours after the last drug injection; food was withdrawn for the last 12 hours.

		Liver		Blood			
				Drug mg%			
Compound	Rat No.	Glycogen mg%	Free drug mg%	Free	Total	Urea N mg%	
Sodium sulfapyridine	183	1330	st. tr.	tr.		18	1
	184	594	tr.	st. tr.		14	
	185	120	”	tr.		16	
	186	910	”	”		16	
	187	244	0	0		14	
Sodium sulfathiazole	195	823	0	0		14	1
	196	1535	0	tr.		24	
	197	726	0	0		18	
	198	1800	0	tr.		18	
	199	458	0	0		18	
Sodium sulfamethylthiazole	180						2
	182	0	126				3
	192	96	19.5	5.7	8	*50*	4
	193	295	tr.	1.4	1.6	14	5
	194	0	44	23	23	*92*	6
Controls: Sodium acetate	200	790				20	
	201	151				16	
	202	185				16	
	203	262				20	
	204	70				20	

tr.—Trace.

st. tr.—Strong trace.

[1] No precipitation in the urinary tract.

[2] Died after 1st injection, kidneys congested.

[3] Sacrificed in dying state 6 hours after 2nd drug injection. Precipitation in renal papillæ, concrements in ureters, liver damage.

[4] Precipitation in renal papillæ, concrements in bladder, bloody urine, liver damage.

[5] Liver and kidneys of normal appearance, no concrement formation.

[6] Precipitation in renal papillæ, concrements in bladder, bloody urine, liver damage.

as evidence of damage to the detoxifying activity of the liver. It seems very probable that the accumulation of high amounts of sulfamethylthiazole is responsible for the degeneration of parenchymatous organs. This possibility is strengthened by the fact that those few animals which showed no lesions in the kidneys after sulfamethylthiazole, and therefore had no retention of the compound in the body (as *e.g.* rat 193 in Table I), failed to develop any signs of liver damage.

TABLE II.
Urinary Findings After a Single Intraperitoneal Injection of 0.5 g/kg Sodium Sulfamethylthiazole in Rats.
(Pronounced hematuria, albuminuria, polyuria and hypostenuria.)

Days after injection	No. of rats	Urine ml per rat	Sp.Gr.	Alb.	Sediment	Drug excretion	
						mg per rat	% acetyl.
0*	5	6	1045	neg.	Amorph. precip.		
1†	5	18	1014	++++	r.b.c. 100§	22	28
2	4	35	1012	+	r.b.c. 50-100	6.5	32
3	4	16	1028	trace	r.b.c. 20-30	1.5	30
4‡	3	10	1038	neg.	single r.b.c.	0.3	

*Control—day before injection.
†1 died: precipitation in renal papillæ, kidney and liver damage.
‡1 died: precipitation in renal papillæ, kidney and liver damage.
§r.b.c.—red blood cells per high power field.

In all other experiments, which will be reported elsewhere in detail, the findings were essentially the same.

Conclusions. Degenerative lesions of liver and kidneys following administration of sulfamethylthiazole are explained as a consequence of the damaging effect of high concentrations of the compound in the body. The accumulation of these toxic concentrations is believed to be due to severe impairment of the renal excretory function caused by precipitation of the acetyl sulfamethylthiazole in the collecting tubules of the kidneys.

11712

Nasogenital Relationship: III, Some Aspects of Sexual Function in Female Rats Deprived of Sphenopalatine Ganglia.

M. C. Shelesnyak, S. Rosen and L. R. Zacharias.
(Introduced by R. T. Frank.)

From the Laboratories of The Mount Sinai Hospital, New York City.

The study of the nasogenital relationship has revealed the production of pseudopregnancy in the female rat following silver nitrate applications to the nasal mucosa[1] and following local anesthetization of the nasal mucosa.[2] The bilateral removal of the sphenopalatine ganglia in adult female rats is followed by pseudopregnancy. This condition is characterized by the persistence of leucocyte cell smears of the vaginal contents, by persistent corpora lutea in the ovary, and by decidual cell reactions to traumatizing of the uterine mucosa. The duration of this state ranges from 8 to 24 days, after which time the regularity of the 4-5 day cycle is resumed.[3]

---

[1] Rosen, S., and Shelesnyak, M. C., Proc. Soc. Exp. Biol. and Med., 1937, **38**, 832.

[2] Shelesnyak, M. C., and Rosen, S., *Endocrinology*, 1938, **23**, 58.

The present report embodies findings from the study of certain sexual functions in female rats which have been deprived of their sphenopalatine ganglia. Series I: The time of vaginal opening in immature rats from which the sphenopalatine ganglia had been removed, was studied in 30 animals. The ganglia were removed from rats ranging in age from 23 to 40 days. This is considerably before the normal time for establishment of the vaginal orifice. Fifty-six littermate controls were given the same care and handling as were the experimental animals, except for the surgical removal of the ganglia. The findings relative to the age and weight of the rats at the time of establishment of the vaginal opening reveal no significant difference in time of vaginal opening between normal and operated animals; mean age and body weight of experimental animals was 54.2±7.8 days and 99.7±14.8 g, and for the control animals the mean age and body weight was 51.8±6.1 days and 98.9±8.2 g.

Series II: The reproductive capacity of rats without sphenopalatine ganglia was tested in 17 cases by matings with virile males. At varying intervals after the removal of the ganglia, ranging from 21 to 189 days post-operative, rats were placed in cages with potent males. Sperm were found in the smears of the vaginal contents of all but 2 cases. In these 2 instances the animals became pseudopregnant; all the remaining animals had litters. The absence of the sphenopalatine ganglia did not interfere with the reproductive behavior of the rat.

Series III: The response of ganglionectomized rats to mechanical stimulation of the os cervix was studied in a group of 253 operated rats. The control group was composed of 190 unoperated animals. Glass rods were used for the mechanical stimulation of the os cervix.[4] (The electrical method of stimulation[5] was not used because the electrical spread may, in the locally anesthetized cervix, move beyond the anesthetized areas.) The animals were stimulated during the proestrous stage of the estrous cycle. One group of animals, and its unoperated control group, were stimulated under deep ether anesthesia; a second group and its control group, under local

---

[3] Rosen, S., Shelesnyak, M. C., and Zacharias, L. R., *Endocrinology*, 1940, **27**, 463.

[4] Long, J. A., and Evans, H. M., *Univ. Calif. Mem.*, 1922, **6**, 1.

[5] Shelesnyak, M. C., *Anat. Rec.*, 1931, **49**, 179.

TABLE I.
Cervical Stimulation of Rats from Which Sphenopalatine Ganglia Are Removed.

Status during cerival stimulation	Non-anesthetized		Topical nupercaine anesthesia (cervix)		Generalized ether anesthesia (deep)	
Type of animal	Control, ganglia intact	Experimental, ganglia absent	Control, ganglia intact	Experimental, ganglia absent	Control, ganglia intact	Experimental, ganglia absent
No. of cases	90	124	50	64	50	65
No. of stimulations (trials)	99	176	100	200	100	203
Ratio C(ases):T(rials)	1:1.1	1:1.4	1:2.0	1:3.5	1:2.0	1:3.1
Pseudo-pregnancy reaction						
No.	73	115	15	30	1	10
% of cases	81	93	30	47	2	15
% of trials	74	65	15	15	1	5

cervical anesthesia (2% nupercaine-Ciba); and a third group without anesthesia. The results and treatments are presented in Table I. The experimental and control animals showed the same reactions to cervical stimulation; and the effectiveness of anesthesia in reducing the number of pseudopregnant responses[6] was comparable in operated and normal animals.

The findings in this study demonstrate that: the establishment of the vaginal orifice, the reproducing capacity, and the pseudopregnancy reaction to cervical stimulation, remain normal in the absence of sphenopalatine ganglia. This suggests that the pseudopregnancy reaction which follows directly after the removal of the sphenopalatine ganglia[3] is related to the immediate effects of ganglion deprivation rather than the absence of the ganglia, and therefore of a transitory nature.

---

[6] Meyer, R. K., Leonard, S. L., and Hisaw, F. L., PROC. SOC. EXP. BIOL. AND MED., 1929, **27**, 340.

11713

Oral Effectiveness of Testosterone Propionate Plus Bile Acid.*

J. A. Wells, M. W. Burrill and R. R. Greene.
(Introduced by A. C. Ivy.)

From the Department of Physiology and Pharmacology, Northwestern University Medical School, Chicago.

Bile acids facilitate the absorption of cholesterol,[1] certain of the fat soluble vitamins,[2-4] and certain drugs[5-7] from the intestinal tract. The similarity in chemical structure between male sex hormones and some of these substances suggests that the administration of bile acids by mouth may enhance the oral activity of the male sex hormone. Such a procedure has been used in the human[8-10] and in the monkey.[11] The studies in the human were such that evaluation of the response to therapy was based largely on subjective factors. The evidence in the monkey was based on the fact that one monkey failed to show postcastration bleeding when treated with testosterone propionate and bile acids by mouth. The purpose of the present study, therefore, was to investigate this question by using a well-established assay method.

Testosterone propionate was the hormone used in the previously quoted reports, and also in this study. This substance is not particularly active by mouth when compared with methyl testosterone, however, it does possess slight oral potency.[12] Desoxycholic acid was the bile acid chosen for this study. This substance has been shown to be the most effective in promoting intestinal absorption of the above mentioned compounds.[1, 2, 6]

---

* We wish to thank Dr. Max Gilbert of Schering Corporation for the testosterone propionate, and Dr. Paul de Haen of Riedel-de Haen for the desoxycholic acid.

[1] Schoenheimer, R., *Biochem. Z.*, 1924, **147**, 258.

[2] Greaves, J. D., and Schmidt, C. L. A., *J. Biol. Chem.*, 1933, **102**, 101.

[3] Greaves, J. D., and Schmidt, C. L. A., Proc. Soc. Exp. Biol. and Med., 1937, **37**, 40.

[4] Cohn, E. T., and Schmidt, C. L. A., Proc. Soc. Exp. Biol. and Med., 1939, **41**, 443.

[5] Wieland, H., and Sorge, H., *Hoppe-Seyler's Z. f. Physiol. Chem.*, 1916, **97**, 1.

[6] Kolda, J., *Compt. Rend. de la Soc. de Biol.*, 1926, **94**, 216.

[7] Langecker, H., *Arch. du Exp. Pharm. and Path.*, 1928, **136**, 257.

[8] Abarbanel, A. R., *Am. J. Ob. and Gyn.*, 1940, **39**, 243.

[9] Silberman, E., Radman, H. M., and Abarbanel, A. R., *Am. J. Ob. and Gyn.*, 1940, **39**, 332.

[10] Abarbanel, A. R., *Endocrinology*, 1940, **26**, 765.

[11] Abarbanel, A. R., Proc. Soc. Exp. Biol. and Med., 1940, **44**, 452.

[12] Miescher, K., and Tschopp, E., *Schwtz. Med. Wchnschr.*, 1938, **68**, 1258.

Male albino rats of 125-150 g in weight were castrated, and oral administration of the various substances was begun on the day after castration. The animals were divided into 4 groups: (1) those receiving 0.1 mg of testosterone propionate per day, (2) those receiving 0.1 mg of testosterone propionate plus 0.5 mg of desoxycholic acid per day, (3) those receiving 1.0 mg of testosterone propionate per day, and (4) those receiving 1.0 mg of testosterone propionate plus 0.5 mg of desoxycholic acid per day.

The oral administration of these substances was continued for 10 days. At the end of this period the animals were sacrificed, and the prostates and seminal vesicles were dissected out and weighed. In the earlier part of the study, 0.6 mg of the bile acid was given per day. However, the animals in this group lost considerable weight and so the dose was reduced to 0.5 mg per day.

It can be seen (Table I) that there is no significant difference between the weights of the prostates in the bile acid treated animals and those which received only testosterone propionate. It can also be seen that the response of the prostate is increased with the increase in dosage of the hormone. There is no great change in the seminal vesicle response, but these structures are known to be less responsive to androgens than the prostates. Therefore, it must be concluded from these data, that desoxycholic acid does not enhance the oral effectiveness of testosterone propionate in the rat. Gastrointestinal absorption is not necessarily the same in the rat as in the human or in the monkey. These results, therefore, do not necessarily apply to these latter animals. However, until more adequate data on the human or monkey is presented, these findings at least cast doubt on the clinical value of combining bile acids with testosterone propionate for oral administration.

TABLE I.
Oral Effectiveness of Test. Prop. Plus Bile Acid.

Dose Test. Prop., mg/day	Dose bile acid, mg/day	No. Anim.	Avg wt Anim., g	Avg wt prostates, mg	Avg wt Sem. V., mg
0.1	None	8	155.2	20.5	41.4
0.1	0.5-0.6	8	130.1	21.4	37.2
1.0	None	9	144.5	42.2	46.3
1.0	0.5	8	144.1	41.2	45.5

11714

Oxygen Requirement of Pathogenic Bacteria Under Starving Conditions.

C. E. Cahn-Bronner. (Introduced by S. A. Levinson.)

*From the Department of Pathology and Bacteriology and the Department of Medicine, University of Illinois, College of Medicine, Chicago.**

The following experiments deal with quantitative changes in the nutrient content of the same kind of media, exposing the bacteria to slight or extensive starvation; but in all these media the bacteria could reproduce continuously, even if they showed morphological changes.

In shake-cultures using agar with decreasing amounts of nutrient broth, *B. coli* will grow as follows: with 100%-50% broth there is anaerobic and aerobic growth; with 20% there is growth only on the surface in a 2 mm thick layer; with 20%-5% broth the superficial layer becomes less dense and gradually deeper, reaching a thickness of 4 mm; with 3% there is no more growth on the surface; below the surface the colonies are very small, becoming gradually larger down to a depth of 5 mm. With 2%-0.1% broth there is no surface growth, but a free zone without any growth below the surface; the bacterial growth forms only a fine, ¼-½ mm thick, dense, line of colonies 3-10 mm below the surface. The poorer the medium, the deeper lies the bacterial layer. The interspace between the surface and the zone of colonies does not show any visible growth. Bacteria do not die there, they are kept alive for several days.

This phenomenon may be more or less general. It was observed in cultures of aerobes like *B. prodigiosus* and *B. pyocyaneus* and in facultative anaerobes like *B. coli, B. typhosus, B. paratyphosus B., B. dysenteriae* Shiga and Flexner, *B. diphtheriae* and *Staphylococcus pyogenes aureus* and *albus,* and in pathogenic organisms as yeasts and actinomyces. These different groups may vary only in the amount of nutritive substance required to produce this phenomenon. Starving pneumococci, enterococci and as far as studied some streptococci do not show this growth in layers, probably because their respiratory system is different.

This zone of bacterial growth formed in shake-cultures under starving conditions is parallel to the surface and follows irregularities of the surface. Air bubbles placed in thin tubes filled with 1.25%

---

*Part of this work was performed in the Instituto di Anatomia Pathologica, University of Pavia, Italy.

agar containing 0.9% nutrient broth show concentric circles around the bubbles formed by colonies after 24 hours' incubation. An isolated colony of fungi located between the surface and a layer of *B. paratyphosus*, may cause an upward directed deviation of the latter layer; probably because the oxygen is partly used up by the fungi.

When the partial tension of oxygen above the medium is increased, the free zone without growth below the surface increases, and the layer of bacterial growth develops in a greater depth. Inversely, when the oxygen tension is decreased, growth appears on the surface in a medium which shows growth only in the depth, when exposed to air. In a medium just rich enough to show surface growth under atmospheric pressure, bacteria reproduce only below the surface, if exposed to increased oxygen tension.

"Bacterial zones" have been described previously by Beijerinck,[1] Jegunow,[2] Lehmann and Curchod,[3] as a chemotactic phenomenon of motile organisms in liquid media. Eisenberg[4] observed ring formation in shake-cultures of spore bearing bacteria in standard media. Braun[5] is the first who mentions bacterial zones in gels of siliciumdioxyd with relation to nutrition, and recently Williams[6] studied bacterial growth in long tubes under high pressure of different gases under different nutrition and obtained similar pictures using oxygen.

To find out which substance may be responsible for this phenomenon, *B. coli* were cultured in a synthetic medium. This medium contains only the indispensable substances for continuous growth for this group[7]: the mineral salts (K_2SO_4, NaH_2PO_4, Na_2HPO_4, $MgSO_4$) and a single source of carbon (lactic acid) and a single source of nitrogen (NH_4Cl). In this type of media the bacteria are forced to a large extent to synthesize, in order to build up their body compounds; in this medium there is no anaerobic growth of facultative anaerobes.[7] Shake-cultures using 1.25% agar containing gradually decreased quantities of this medium show a fine line of growth situated gradually deeper below the surface. When only the source of nitrogen is reduced and the source of carbon is kept constant, then this phenomenon does not appear. When the carbon compound which is also the main source of energy, is reduced, and the nitrogen compound is kept constant, then the characteristic structure of layer formation occurs.[5] Thus the increased sensibility against oxygen is due to a lack of carbon compound, in other words, a lack of an easily oxidizable substance or an adequate oxygen acceptor. The less the medium contains of this substance, the more inhibitory is the action of oxygen on the organism, or may be the action of a substance formed by the bacteria in the presence of oxygen.

---

[1] Beijerinck, M. W., *CBl. f. Bakt.*, 1893, 14.

[2] Jegunow, *CBl. f. Bakt.*, 1898, **2**, 4.

[3] Lehmann and Curchod, *CBl. f. Bakt.*, 1905, **2**, 14.

[4] Eisenberg, *CBl. f. Bakt. I*, 1919, **82**, 209.

[5] Braun, H., *CBl. f. Bakt. I*, 1931, **122**, 5.

[6] Williams, J. W., *Growth*, 1939, **3**, 21.

[7] Braun, H., and Cahn-Bronner, C. E., *CBl. f. Bakt.*, 1921, **86**, 380; *Biochem. Z.*, 1922, **131**, 272.

The distance of this thin layer of colonies from the surface varies with the temperature; it increases when the incubator-temperature of 37° is lowered to 22, 17, 12° or when it is raised above the optimal temperature of 37° to 42°. Exposure to sunlight also increases the free zone below the surface and the inhibitory action of oxygen; if a shake-culture is partially darkened by black paper, the bacterial layer develops closer to the surface in the darkened part, more distant from the surface in the exposed part of the agar column.

After some time of incubation under starving conditions 2, 3, sometimes up to 12 layers are formed below the uppermost layer which itself is distant from the surface. Each layer is separated from the one above by a narrow free zone. The number of these layers and the width of this formation is in relation to the amount of nutrient in the medium, the number of inoculated organism, and the temperature.

The unequal distribution of oxygen in the media is also an important factor in this ring-formation. The first layer forms by its respiration with extensive oxygen-intake a barrier against the diffusion of oxygen downwards. In the meantime the small quantity of nutrient which is present in the neighborhood of these colonies under starving conditions is partly used up, and inhibitory products are accumulated, thus a small free zone without growth develops beneath this layer and the next layer is formed after some time in a marked distance from the upper layer. This oxygen barrier of bacterial zones and the distribution of oxygen in colloidal media will be demonstrated in a subsequent report.

Conclusions and Summary. Under partial starvation some aerobic and facultative anerobic bacteria, yeasts, and fungi grow only in a fine, dense layer below the surface of the shake-cultures where the relation between nutrient-content and oxygen is an optimum. Under increasing starvation bacteria become increasingly sensitive against oxygen. Oxygen causes a bacteriostatic effect, the rate of which is in relation to the nutrient content of the medium. The less the medium contains of carbon compounds which may act as oxygen acceptor, the more inhibitory is the action of oxygen on the organisms. Variation in temperature below and above the optimal temperature and exposure to light increases the sensibility against oxygen.

11715

Prevention of Nutritional Myopathy of Ducklings by α-Tocopherol.

Alwin M. Pappenheimer.

From the Department of Pathology, College of Physicians and Surgeons, Columbia University, New York City.

The author of this paper with M. Goettsch described a nutritional disease of Pekin ducklings characterized by extensive degeneration of the voluntary muscles.[1] The disease was produced by the same diet (108) which in chicks brought about necrosis of the central nervous system (nutritional encephalomalacia). It has now been shown that this chick disease is a manifestation of vitamin E deficiency, definitely preventable by wheat germ oil and other vegetable oils, or their non-saponifiable fractions,[2] and by pure vitamin E in the form of natural or dl-α-tocopherol.[3, 4]

Preliminary experiments indicated that soy bean oil and wheat germ oil in adequate dosages protected ducklings against the development of muscle degeneration. Conclusive proof that the muscle disease is attributable to lack of vitamin E has not yet been given, and it is the purpose of this short communication to fill this gap in our knowledge.

Experimental. One day old white Pekin ducklings obtained from Sears Roebuck & Co. were given a commercial starting mash for several days, and then placed upon the experimental diet 108.* They were kept for about 2 weeks in battery brooders and then in individual wire cages. Dl-α-tocopherol was dissolved in sesame oil, the solution having been standardized so that with the pipette used, 1 drop was equivalent to 1 mg of α-tocopherol. It was administered daily in gelatin capsules. The controls received the same amount of

*Diet 108:

Skimmed Milk Powder	15.0
Casein (Merck's Technical)	20.5
Cornstarch	20.0
Lard	21.0
Cod Liver Oil (Mead Johnson)	2.0
Yeast (Fleischmann's Baker)	3.0
Salt Mixture (McCollum 185)	8.5
Paper Pulp	10.0

---

[1] Pappenheimer, A. M., and Goettsch, M., *J. Exp. Med.*, 1934, **59**, 35.

[2] Goettsch, M., and Pappenheimer, A. M., *J. Biol. Chem.*, 1936, **114**, 673.

[3] Dam, H., Glavind, J., Bernth, O., and Hagens, E., *Nature*, 1938, **142**, 1157.

[4] Pappenheimer, A. M., Goettsch, M., and Jungherr, E., *Storrs Agric. Exp. Sta.*, 1939, Bull. 229.

sesame oil without α-tocopherol. A complete autopsy was performed, and at least four pieces of skeletal muscle examined microscopically. The animals were killed when they exhibited definite symptoms of muscular weakness. The survivors were kept under observation for about 5 weeks and then killed by decapitation.

Gizzard erosions were an annoying complication, and several ducklings succumbed to perforating ulcers. Many of the animals also suffered from slipped tendon (perosis), in spite of the fact that the manganese content of the salt mixture in diet 108 in the first experiment was increased to 0.1%. Following the report of Jukes,[5] of the preventive effect of cholin upon perosis in turkeys, 1-3 drops of a millimolar solution of cholin acetate were given daily by pipette to one group of 12 ducklings during the first 2 weeks of the experiment. Only 2 of this group developed the deformity, in contrast to 10 of 13 in the control groups. Unfortunately, it was not possible to continue the administration of the cholin acetate throughout the experiment; it seems, however, to have exercised a distinctly protective effect.

Experiment I. In this experiment, 13 ducklings received daily 1 mg of dl-α-tocopherol acetate in sesame oil; 12 controls, 1 drop (approximately 1 mg) of sesame oil. The results are shown in Table I.

It is obvious that a daily dose of 1 mg of α-tocopherol did not

TABLE I.
Effect of α-Tocopherol upon Nutritional Myopathy.

Group A 1 mg dl-α-tocopherol acetate daily					Group B 1 drop of sesame oil daily				
No.	Days on diet	Total dosage, mg	Gross	Micr.	No.	Days on diet	Total dosage, mg	Gross	Micr.
4501	16	17	—	—	4515	16	17	+	++
4502*	15	15	—	—	4516†	23	20	?	+++
4504	D20	20	—	±	4517	14	14	++++	++++
4505	29	28	+++	++++	4518	27	27	—	±
4506*	19	20	—	—	4519	27	25	—	++
4507	31	31	—	+‡	4520	27	25	++	+++
4509	31	31	++	+++	4521	20	20	±	±
4510	31	31	—	+	4522	12	16	—	—
4511*	D14	14	—	+	4523	10	11	—	—
4512	31	31	—	±	4524	20	20	+++	++++
4513	20	21	—	++	4525	27	27	+	++
4514	D22	22	—	++	4527	27	26	—	+++‡
4580	29	28	+++	++++	4528	19	20	++++	++++

*Ulcers of gizzard.
†Killed by rat.
‡Healing.

---

[5] Jukes, T. H., *J. Biol. Chem.*, 1940, **134**, 789.

TABLE II.
Effect of α-Tocopherol upon Nutritional Myopathy.

Group A dl-α-tocopherol					Group B† Oil of sesame				
No.	Days on diet	Total dosage, mg	Gross	Micr.	No.	Days on diet	Total dosage, drops	Gross	Micr.
4530	31	98	—	—	4547	16	46	—	+§
4531*	D6*	14	—	—	4545	24	74	++++	++
4532	33	110	—	—	4549	27	74	++++	+++
4535	33	110	—	—	4550	24	82	++++	++++
4536	K6	12	—	—	4551	10	26	++++	++++
4537	17	50	—	—	4552	16	46	++	++++
4538	16	46	—	—	4553	28	106	++++	++++
4541	31	110	—	±	4554‡	17	50	—	—
4542	17	50	—	—	4548	28	102	+++	+++
4543	34	118	—	—	4556	11	26	++++	+++
4546*	18	56	—	—	4558	11	30	±	+++
4555	17	52	—	—	4557	34	98	++	++++
4598	34	118	—	—					

*Gizzard ulcer.
†Cholin acetate (1-3 gtt.) for 16 days.
‡Escaped from cage—killed before development of symptoms.
§Healing.

prevent the development of muscle degeneration. The experiment was therefore repeated with larger doses; 2 mg of dl-α-tocopherol acetate daily for the first 9 days, then 4 mg of dl-α-tocopherol daily until the end of the experiment. This amount was found to afford complete protection, as is shown in Table II.

Excluding ducks 4531 and 4536 which died of incidental causes before the period during which muscle lesions develop, all the animals of group A receiving the larger doses of α-tocopherol were completely protected. The controls receiving only an equivalent amount of sesame oil developed the disease in characteristic fashion, the only exceptions being ducks 4547 and 4554 which were killed prematurely.

Conclusion. The nutritional myopathy of ducklings which develops on vitamin E deficient diet is completely prevented through the experimental period by daily administration of 4 mg of synthetic α-tocopherol. A dosage of 1 mg daily does not afford protection.[†]

[†] We are indebted to the Hoffman-LaRoche Co. for the synthetic α-tocopherol. acetate used in the experiments, and to Merck & Co. for the dl-α-tocopherol.

11716

Studies with Antigens. IV. Purification of Extracts of Grass Pollens.

C. H. Boatner and B. G. Efron. (Introduced by I. Cohn.)

From the Department of Chemistry, Newcomb College, Tulane University, and the Division of Allergy, Touro Infirmary, New Orleans, La.

We have reported[1, 2] fractionation methods for the purification and concentration of the house-dust allergen. Solutions of purified house-dust produce specific positive scratch reactions in house-dust-sensitive patients in concentrations of 0.50 to 0.87%, and specific intracutaneous reactions in dilutions of 1/50,000 to 1/50,000,000.[3]

The technic has now been extended to the purification and concentration of grass-pollen allergens. (*Phleum pratense, Capriola dactylon, Paspalum dilatatum, Holcus halapensis.*)

Comparative skin reactions which were used for evaluating the effectiveness of all fractionations were made with equal concentrations of extracts. The significance of differences in skin reactions induced by them were estimated by the chi-square statistic.[4]

Aqueous extracts of defatted pollens were subjected to fractional precipitation by the addition of water-miscible organic solvents such as dioxane, acetone, isopropyl alcohol. The fractionation-technic is briefly described as follows: The precipitate which forms when a small amount of organic liquid is added to the aqueous extract is removed, and then an additional amount of organic liquid is added to the filtrate; this procedure is repeated to give progressively larger proportions of organic liquid with successive separations of insoluble fractions. The fractionations with organic liquids are carried out at 5°-7° C.

For purposes of brevity, our description will be limited to the results obtained by the use of dioxane.

The reactivity of the fractions precipitated by concentrations of dioxane less than 25%, or greater than 75% was found to be slight and significantly less than that of the original extracts from which they were precipitated; the fractions precipitated by 25 to 75% concentrations caused marked reactions, which were significantly greater than those induced by the original extracts.

---

[1] Boatner, C. H., Efron, B. G., and Dorfman, R. I., *Science*, 1940, **91**, 389.

[2] Boatner, C. H., Efron, B. G., and Dorfman, R. I., *Allergy*, to be published.

[3] Efron, B. G., Boatner, C. H., and Pabst, M. R., *Investigative Dermatology*, to be published.

[4] Pabst, M. R., Boatner, C. H., and Efron, B. G., *N. O. Med. and Surg.*, 1940, **93**, 142.

Dialysis experiments showed that No. 1200 cellophane membrane is permeable to much of the inert material in the extracts, but practically impermeable to the allergens. It permitted the passage of dialyzable material of very little potency.

The skin reactivity of extracts fractionated with dioxane followed by dialysis was significantly greater than that of original extracts dialyzed over the same period of time. Therefore, the allergenically inert material removed by fractionation with dioxane is non-dialyzable.

Extracts purified by fractionation with dioxane were treated with high concentrations of salts, such as ammonium, sodium, and zinc sulfate. In the use of ammonium sulfate, 60 g of the salt were added to each 100 ml of the extract. The precipitate was dissolved in water, and again treated with ammonium sulfate. The salt adhering to the precipitate was removed by dialysis.

The skin reacting potency of the extract treated by fractionation with dioxane, with ammonium sulfate, and then by dialysis was significantly greater than that of the extract purified by fractionation with dioxane and by dialysis. The extracts compared had been subjected to dialysis against running water for the same length of time.

Table I compares the results of cutaneous tests of the original extract of timothy pollen and of the extract purified by fractionation with dioxane, ammonium sulfate, and dialysis. These data show that the potency of the purified extract is significantly greater than that of the original extract.

TABLE I.
No. of Reactions.

	Equal-sized	Larger	Smaller	Total
Original timothy-extract	11	1	46	58
Purified timothy-extract	11	46	1	58

$\chi^2 = 90.194$, $n = 2$, $P =$ less than .01.

Results similar to those shown in Table I were obtained with the other grass-pollens studied.

The increased potency of the purified extracts is due to the purification of the allergens resulting in their increased concentration per unit-weight of dissolved material in the extracts. This conclusion is warranted by the following observations: Scratch tests performed on individuals who had no allergic disease failed to show any evidence of irritation when tests were made with the various fractions of the extracts. Patients who suffered from allergic disease but who were not clinically sensitive to grass-pollen failed to show positive reactions to scratch tests with the various fractions. Patients who were clinically sensitive to grass-pollens showed significantly larger skin reactions with the more highly purified extracts than with the less highly purified or with the untreated extracts.

Summary. 1. Fractionation of aqueous grass-pollen extracts with water-miscible organic liquids promotes purification of these extracts. 2. Precipitation of extracts purified by the above treatment with high concentrations of sulfates affords further purification.

11717 P

Effect of Gramicidin Suspended in Mineral Oil on Streptococci of Bovine Mastitis.

R. B. LITTLE, R. J. DUBOS AND R. D. HOTCHKISS.
From the Rockefeller Institute for Medical Research.

Gramicidin, an alcohol-soluble, water-insoluble substance extracted from cultures of an aerobic sporulating bacillus, has been found to exert a marked bactericidal effect against Gram positive microörganisms, both *in vitro* and *in vivo*.[1] When injected by way of the cistern into infected quarters of cows suffering from mastitis caused by *Streptococcus agalactiae* (Lancefield serological group B), gramicidin apparently cured a number of the infections.[2]

The method originally used for the administration of gramicidin in the treatment of bovine mastitis consisted in diluting 60-240 mg of the bactericidal substance in 1000 cc of aqueous media and injecting this material into the cistern of the infected quarter. As reported earlier, this method of administration caused an intense swelling of the treated quarter, accompanied by a marked elevation of temperature. Although this reaction lasted only a few hours, it was severe enough to prevent repeated treatments on successive days.

It has now been found that sterile mineral oil is a suitable, non-irritating vehicle for the administration of gramicidin. Of 42 quarters treated with gramicidin-oil mixtures, only 4, which had received 120-160 mg of bactericidal substance, exhibited a severe reaction. On the second and third day following treatment, the fore milk in 3 of these quarters was tinged with blood, whereas the secretion of milk was suppressed for 4 days in a fourth quarter. The reaction in the remaining 38 quarters was milder than in those injected with gramicidin in water.

---

[1] Dubos, R. J., *Ann. Int. Med.*, 1940, **13**, 2025.

[2] Little, R. B., Dubos, R. J., and Hotchkiss, R. D., PROC. SOC. EXP. BIOL. AND MED., 1940, **44**, 444.

On the basis of present experience, the following technique of treatment appears the most satisfactory. 2-3 cc of an alcoholic solution of gramicidin, containing 40 mg per cc, are emulsified in 15 cc of sterile double distilled water and the emulsion mixed thoroughly in 25 cc of heavy mineral oil. The gramicidin-oil mixture is injected into the cistern shortly after the morning milking and allowed to remain until the evening milking. In usual cases, the mildness of the reaction makes it possible—if necessary—to repeat the treatment for several days in succession or to treat more than one quarter at the same time.

The effect of the treatment has been determined by a combination of bacteriological tests including plating the fore milk on blood agar, direct incubation of the milk sample, and the Hotis test. These tests were made repeatedly over periods of more than 30 days and attempts were made to recover and identify the cultural and serological types of streptococci responsible for the mastitis.

Of 31 quarters naturally infected with *Streptococcus agalactiae* and treated by the gramicidin-oil mixture, 26 appear to have responded by a complete disappearance of the streptococci. The infection in some of the cured cases was of a severe chronic nature. In 20 of the quarters a single treatment sufficed, while 6 required additional treatments. The infection was not eliminated in 5 quarters.*

Surprisingly enough, less satisfactory results have been obtained in the treatment of bovine mastitis caused by *Streptococcus uberis;* of four cases of this condition which were treated with gramicidin-oil mixtures, only one responded satisfactorily. The nature of this discrepancy is under investigation.

---

* In the case of 8 infected quarters which were treated with mineral oil alone, without the addition of gramicidin, we have observed a temporary reduction or even disappearance of the streptococci present in the milk. Nevertheless, in these 8 cases the number of bacteria returned to the original level 2-3 days after the last injection of oil alone; it is possible however that this treatment may be sufficient at times to cure an infection of a very mild character.

11718

Demonstration of Rapid Production of Lactic Acid in Oral Cavity.*

ISAAC NEUWIRTH AND JULIUS A. KLOSTERMAN.

From the Departments of Pharmacology and Therapeutics and Bacteriology, College of Dentistry, New York University, New York City.

Despite the opposing schools of thought on the origin of dental caries, it is generally agreed that one of the important factors in this condition is a solution of the enamel by acids produced by microorganisms in the oral cavity. Many *in vitro* studies have been made showing the production of acids by mouth organisms over periods of time ranging from an hour to days or more.

Except for the work of Stephan[1] recently reported, we know of no work which shows the quick production of acids in the mouth. Stephan reports that, immediately following rinsing of the mouth with certain carbohydrate solutions, there is a marked fall in the pH of plaques and open tooth cavities in the mouth. What particular acid or acids are responsible for this fall in pH is not stated.

In the work herein reported, the rapid production of lactic acid in the oral cavity is shown as the result of allowing certain carbohydrates to dissolve in the mouth. This same finding is also demonstrated *in vitro,* by incubating such carbohydrate solutions with saliva, but not if the saliva has first been rendered sterile by passing it through a Seitz filter.

Our work was made possible only through having at hand the Miller and Muntz method[2] for estimating ultramicroquantities of lactic acid.† The method was modified to adapt it for use with the

---

* Aided by a grant from the Bristol-Myers Co., New York, N. Y.

A preliminary report of part of this work was made before the New York Section, International Association for Dental Research, January 30, 1940. Published in *J. Dent. Res.*, 1940, **19**, 193.

[1] Stephan, R. M., *J. Am. Dent. Assn.*, 1940, **27**, 718.

[2] Miller, B. F., and Muntz, J. A., *J. Biol. Chem.*, 1938, **126**, 413.

† Pyruvic acid has recently come into prominence as one of the acids involved in the production of dental caries.[3,4] If the time of heating in the Miller and Muntz method for lactic acid is 5 minutes, pyruvic acid, if present, will interfere. However, this interference is eliminated if the time of heating is 15 minutes. Some preliminary experiments indicate that in our work no pyruvic acid is present in the oral cavity contents when the criterion set up by Miller and Muntz is used—*i. e.*, comparing the findings for lactic acid when the solutions are heated for 15 minutes instead of 5 minutes. However, if the method using 2-4-dinitrophenylhydrazine as given by Lu[5] is used, there is evidence of pyruvic acid in our control and experi-

Summerson photoelectric colorimeter[6] by adding 4 cc of concentrated sulfuric acid before making the readings.

The procedure of our experiments may be briefly stated as follows: In the *in vivo* experiments, the mouth was rinsed 3 times with 25 cc quantities of water, each rinsing lasting 20 seconds. Ten minutes later, the first control sample of saliva was taken, and the second, 10 minutes after the first. A tablet of carbohydrate (containing about 1.5 g carbohydrate) was then allowed to dissolve in the mouth.‡ Ten minutes after the tablet had completely dissolved, the next sample of saliva was obtained. Other samples followed at 10 minute intervals. The period of collecting samples was usually 1½ minutes, the amounts varying from 0.5 cc to 1.7 cc. Analyses for lactic acid were made.

In the *in vitro* experiments, saliva was obtained as for the first control sample noted above and mixed with an equal quantity of approximately 0.1% carbohydrate solution. This mixture was incubated at 38° and samples for analysis taken at 10 minute intervals. In some instances, besides making determinations for lactic acid, sugar was determined by the Benedict method.[7]

Our subjects were laboratory workers, all males except Subject 1. Their ages ranged from 22 to 42 years.

The results of the *in vivo* experiments are shown in Table I. It is quite evident that within 10 minutes after banana or tablets of

---

mental samples. The amounts found are, however, too small to account for very much of the lactic acid obtained in our work. We are investigating this further.

[3] Fosdick, L. S., and Hansen, H. L., *J. Am. Dent. Assn.*, 1936, **23**, 401.

[4] Fosdick, L. S., Hansen, H. L., and Wessinger, G. D., *J. Am. Dent. Assn.*, 1937, **24**, 1445.

[5] Lu, G. D., *Biochem. J.*, 1939, **33**, 249.

[6] Summerson, W. H., *J. Biol. Chem.*, 1939, **130**, 149.

‡ Tablets of sucrose, glucose, and starch were used. The time for complete solution and disappearance of these from the mouth varied from 4 to 7 minutes. Acacia tablets were also used, but the results obtained with these are not considered reliable. In some of these acacia experiments, slight but significant increases of lactic acid were found. These were due to a peculiar blue color given in the reaction. That these increases were not due to lactic acid is shown by the fact that no such results were obtained when acacia was used in the *in vitro* experiments. The peculiar blue color also occurred when the reaction used in this work was carried out with pure acacia or arabinose solutions.

In contrast to the 1.5 g tablets of the carbohydrates used in the *in vivo* experiments, about 0.7 g of gelatin, in the form of small thin sheets, was used. This was allowed to dissolve in the mouth for about 8 minutes; at this time, not all of the gelatin had completely dissolved.

It took about a minute for 10 g of banana to completely dissolve in the mouth.

[7] Benedict, S. R., *J. Biol. Chem.*, 1931, **92**, 141.

TABLE I.

Production of Lactic Acid in the Oral Cavity.

Results are expressed as mg of lactic acid per 100 cc of saliva. Samples are taken at 10-minute intervals, samples 1 and 2 serving as controls. Sample 3 is taken 10 minutes after the complete solution of the test material in the mouth (see text and footnote ‡).

		Sample					
Subject	Test Substance	1	2	3	4	5	6
1	Glucose	1.0	1.0	19.5	2.9	2.0	1.7
2	”	2.3	1.6	50.0	7.7	1.9	1.4
3	”	3.0	3.8	33.4	8.4	4.7	3.6
4	”	3.4	2.4	14.0	4.3	2.3	2.2
5	”	2.7	3.7	15.4	5.6	4.5	2.3
1	Sucrose	1.3	1.0	15.9	6.0	1.5	1.2
2	”	2.5	1.8	43.0	15.7	3.2	3.2
3	”	2.6	3.2	40.0	10.8	5.0	3.3
4	”	2.0	1.8	16.6	4.8	3.5	2.0
5	”	2.8	2.5	26.7	10.4	5.8	5.1
1	Starch	1.6	1.3	4.2	1.4	1.2	1.3
2	”	1.9	1.7	13.6	3.1	2.0	1.5
3	”	2.4	2.2	10.2	3.7	2.6	1.8
4	”	3.0	2.5	12.0	3.9	2.3	2.5
5	”	2.3	2.1	13.1	3.3	3.3	2.8
1	Gelatin	2.0	1.9	1.7	1.6	1.6	1.7
2	”	2.7	2.1	2.8	2.4	2.1	1.8
3	”	3.2	2.9	3.0	2.8	2.8	2.6
4	”	3.6	3.3	3.3	3.2	2.4	2.8
5	”	2.9	2.4	2.5	2.8	2.8	3.0
1	Banana	1.0	1.4	10.0	2.2	1.8	1.1
2	”	1.8	1.5	31.6	3.7	1.6	1.8
3	”	2.2	2.5	15.3	5.7	3.9	3.7
4	”	3.2	2.6	11.7	4.0	2.9	2.6
5	”	2.8	2.4	24.2	5.8	4.1	4.1

sucrose, glucose or starch have been completely dissolved in the mouth, there is a marked increase of lactic acid in the contents of the oral cavity. After this, the concentration of lactic acid falls off sharply. The increases noted cannot be ascribed to changes in the rate of salivary secretion.

Table II illustrates the effect on lactic acid production of incubating mixtures of saliva and various substances. As shown in the *in vivo* experiments, increases in lactic acid are shown with starch and sucrose, but not with gelatin. Negative findings in these experiments are also given with inulin, arabinose and acacia.

That the production of lactic acid from carbohydrates in the *in vitro* experiments is accompanied by a loss of carbohydrates is shown in Table III.

Where saliva has been rendered sterile by passing it through a

TABLE II.

Production of Lactic Acid *in vitro*.

Results are expressed as mg of lactic acid per 100 cc of mixture, composed of equal parts of saliva and 0.1% test substance solution. Samples are taken at 10-minute intervals, sample 1 being the control taken immediately after making the mixture.

Saliva from Subject	Test Substance	Sample					
		1	2	3	4	5	6
1	Sucrose	1.2	3.3	6.7	10.2	14.8	17.8
2	,,	6.6	9.1	12.6	16.1	21.0	26.4
1	Starch	1.0	1.7	3.7	6.0	8.2	11.2
2	,,	2.2	3.2	4.4	6.5	9.2	12.3
1	Inulin	1.9	1.7	1.5	1.4	1.3	1.2
5	Acacia	5.5	5.5	5.3	5.0	4.7	4.4
2	Arabinose	2.0	1.6	1.3	1.0	0.8	0.6
1	Gelatin	1.2	1.0	0.8	0.8	0.8	0.8

TABLE III.

Production of Lactic Acid *in vitro*.

Results are expressed as mg of lactic acid and mg of the carbohydrate (shown in parenthesis) per 100 cc of mixture, composed of equal parts of saliva and 0.1% carbohydrate solution. Samples are taken at 10-minute intervals, sample 1 being the control taken immediately after making the mixture.

Saliva from Subject	Test Substance	Sample					
		1	2	3	4	5	6
4	Xylose	1.2(50)	1.2(50)	1.3(49)	1.3(51)	1.3(50)	1.2(51)
1	Glucose	0.9(53)	1.9(49)	4.0(44)	6.9(38)	9.6(32)	13.2(25)
2	,,	2.9(53)	5.6(47)	8.8(38)	11.6(28)	15.2(19)	19.4(10)

Seitz filter, and then incubating such saliva with sucrose or glucose solutions, no production of lactic acid is obtained. The Seitz filtrates contain ptyalin. The production of lactic acid in our experiments must therefore be ascribed to the action of microorganisms.[§]

Summary. The rapid production (within 10 minutes) of lactic acid in the oral cavity and *in vitro* is demonstrated as the result of the action of oral microorganisms on certain carbohydrates.

[§] The point may be raised that the presence of certain carbohydrates in the mouth may stimulate the salivary glands to increased lactic acid production. However, the negative findings with acacia (and gelatin) in the *in vivo* experiments and with sterile saliva in the *in vitro* experiments tend to negate such a contention. It may be pointed out here that in connection with another problem being carried out in collaboration with Dr. W. H. Summerson at Cornell University Medical College, it has been found that there is a marked increase in lactic acid in paraffin-stimulated saliva as compared to lactic acid in resting saliva. However, if paraffin is chewed vigorously for 5 or 6 minutes, and a sample of saliva is obtained 10 minutes later, no increases in lactic acid as reported in this paper are found. The findings on lactic acid content of stimulated and non-stimulated or resting salivas will be reported shortly.

11719 P

Changes in Cerebral Blood Flow and Arterio-Venous Oxygen Difference During Insulin Hypoglycemia.*

H. E. Himwich, K. M. Bowman, C. Daly, J. F. Fazekas,
J. Wortis and W. Goldfarb.

From the Department of Physiology and Pharmacology, Albany Medical College, Union University, Albany, N. Y., and Psychiatric Division, Bellevue Hospital, New York City.

Previous work has disclosed that the arterio-venous oxygen difference of cerebral blood is diminished during insulin hypoglycemia.[1, 2] However, the arterio-venous oxygen difference is determined not only by the metabolic rate of the brain, but also by velocity of blood flow. Therefore, the possibility exists that the smaller oxygen difference found may be due to a more rapid blood flow during hypoglycemia. Observations by Loman and Myerson[3] on human subjects and Leibel and Hall[4] on rabbits, have failed to demonstrate any marked changes in the rate of cerebral blood flow during insulin hypoglycemia uncomplicated by convulsions. However, for conclusive information regarding changes of brain metabolism, it is necessary to determine, simultaneously, the cerebral blood flow and the arterio-venous oxygen difference. The results of such a study on patients with schizophrenia are presented in this preliminary report.

Methods for the collection and analysis of the blood samples have been previously described.[2] The rate of blood flow in the internal jugular vein was estimated by a modification of the Gibbs thermostromuhr. This instrument proved to be at least as sensitive as the original and will be described elsewhere. Observations on the blood flow were made over long periods during which the position of the thermostromuhr in the vein was maintained constant. A special technique was devised which prevented error resulting from the formation of clots on the needle.

In 5 cases the blood flow and the arterio-venous differences were determined before injection of insulin as well as throughout the

---

* Aided by a grant from the Child Neurology Research (Friedsam Foundation).

[1] Dameshek, W., Myerson, A., and Stephenson, C., *Arch. Neurol. and Psychiat.*, 1935, **33**, 1.

[2] Himwich, H. E., Bowman, K. M., Wortis, J., and Fazekas, J. F., *J. Nerv. and Ment. Dis.*, 1939, **89**, 273.

[3] Loman, J., and Myerson, A., *Am. J. Psychiat.*, 1936, **92**, 791.

[4] Leibel, B. S., and Hall, G. E., Proc. Soc. Exp. Biol. and Med., 1938, **38**, 894.

TABLE I.
Arterio-Venous Oxygen Differences and Cerebral Blood Flow During Iusulin Hypoglycemia.

Patient	Glucose Art.	Oxygen			Observed change of blood flow	Calculated* change of blood flow
		Art.	Ven.	Diff.	%	%
1	83	17.11	10.97	6.14		
	19	17.35	13.28	4.07	—10	+ 51
	15	17.83	15.67	2.16	—30	+184
	17	17.63	15.30	2.33	—10	+168
	13	17.52	16.18	1.34	0	+358
2	34	16.47	14.93	1.54		
	117	16.62	11.30	5.32	—20	—246

*Calculated from A:V oxygen difference if brain metabolism is unchanged.

course of the coma and the subsequent administration of glucose. In 3 other instances the observations were begun during coma and continued until after arousal with intravenous glucose. The data observed in one of each of these 2 types of experiments are presented in the table. In none of these experiments could the low arterio-venous difference during hypoglycemia be accounted for by any change in blood flow, and in most cases the changes in blood flow were opposite in direction, showing a slow blood flow with a low arterio-venous difference.

Thus, the decreased arterio-venous difference of oxygen cannot be attributed to a more rapid blood flow, but must be due to a diminished brain metabolism. These results are in accordance with the conception that the chief fuel of the brain is glucose.[2] The fact that the cerebral respiratory quotient is unity;[5] that glucose awakens patients from hypoglycemic coma; and that the alpha waves of the electroencephalograph disappear in hypoglycemia and reappear after the administration of glucose[6, 7] all indicate the importance of glucose in brain metabolism.

---

[5] Himwich, H. E., and Nahum, L. H., *Am. J. Physiol.*, 1932, **101**, 446.

[6] Hoagland, H., Rubin, M. A., and Cameron, D. E., *Am. J. Physiol.*, 1937, **120**, 559.

[7] Himwich, H. E., Hadidian, Z., Fazekas, J. F., and Hoagland, H., *Am. J. Physiol.*, 1939, **125**, 578.

11720

Some Factors Involved in the Assay of Renin.

J. W. Remington, W. D. Collings,* H. W. Hays and W. W. Swingle.

From the Section of Physiology, Biological Laboratory, Princeton University, Princeton, N. J.

An assay of renin on anesthetized dogs has proved difficult because of the variable sensitivity of individual animals.[1, 2] Assays based on fewer than 10 tests have not always proved reliable. The factors underlying this difference in sensitivity are not wholly clear. Since only one test was made on an individual dog in one day, and an interval of 4-5 days allowed between tests, it is not plausible that a tachyphylaxis or a progressive resistance to the renin was contributing to the variability. Data has been assembled on several points which might bear on the inconstancy of assay results. Does the level of the starting blood pressure, which shows variation in anesthetized dogs, affect the magnitude of the response to renin? Would an assay based on a minimum number of dogs with repetition of tests on each be more reliable? Since it has been reported[3] that nembutal anesthesia reduces and even eliminates the response of the rabbit to renin, to what extent is the anesthetic affecting the results obtained with dogs?

I. *Influence of the Starting Pressure on the Response to Renin.* The results of 200 assay tests, covering some 20 lots of extract irrespective of differences in potency, have been grouped according to the initial blood pressures of the dogs used. Although these pressures varied from 80 to 160 mm Hg, over 80% were between the limits of 95 and 130 mm Hg, which can be considered the normal range.[4] All renin doses of 0.3 mg per kg body weight or more gave blood pressure responses not significantly different from each other. These maximal responses have been grouped together in the overdosage category.

Table I shows that an inverse correlation exists between the blood pressure rises evoked by overdosages of renin and the starting

---

* E. R. Squibb and Sons Fellow in the Biological Sciences.

[1] Swingle, W. W., Taylor, A. R., Collings, W. D., and Hays, H. W., *Am. J. Physiol.*, 1939, **127**, 768.

[2] Wakerlin, G. E., and Chobot, G. R., *Am. J. Physiol.*, 1939, **126**, 646.

[3] Pickering, G. W., and Prinzmetal, W., *Clin. Sci.*, 1938, **3**, 211.

[4] These represent mean pressures, taken by the needle puncture method (Parkins, W. M., *Am. J. Physiol.*, 1934, **107**, 518).

TABLE I.
Correlation Between Initial Blood Pressure and Response to Renin in the Anesthetized Dog.

Initial blood pressure mm Hg	Overdosage			0.2 mg/kg body wt			0.1 mg/kg body wt		
	No. of tests	Blood pressure rise mm Hg	Blood pressure peak mm Hg	No. of tests	Blood pressure rise mm Hg	Blood pressure peak mm Hg	No. of tests	Blood pressure rise mm Hg	Blood pressure peak mm Hg
81- 90	7	125±3.7	211±3.8	1	93	184	2	68	157
91-100	19	119±3.2	209±3.1	8	84±7.6	177±5.6	11	53±2.8	150±3.3
101-110	30	104±2.5	212±2.4	10	72±3.2	177±3.0	9	51±5.4	159±5.3
111-120	32	92±2.4	213±2.3	8	68±2.9	183±2.8	7	42±3.3	159±2.9
121-130	12	79±4.5	207±3.4	6	67±3.5	192±3.3	11	46±2.7	169±2.3
131-140	4	77	213	9	46±2.8	183±2.8	6	31±3.1	165±2.9
141-150	1	60	208	4	43	187	3	37	180
Avg	105*	101±1.7	211±1.5	46*	65±2.2	183±1.7	49*	47±1.5	161±1.6

*Totals.

pressure levels. In other words, the maximum rise is limited by a fairly constant maximum peak pressure. Such a clear correlation between rise and starting pressures is not so evident with the lower doses of renin. There is some indication that the rise decreases as the initial pressure becomes greater, but within the normal starting pressure range the trend is not sufficient to warrant correcting assay results for these differences. In most groups the peak pressures obtained show less variability than do the rises. With an assay based on peak pressures, too, corrections probably need not be made for differences in initial pressures.

II. *Effect of Repetition of Renin Tests on the Same Dog.* A single lot of renin extract, containing 5.1 mg solids (0.8 mg N) per cc, was used for repeated tests on 6 dogs. An interval of at least 72 hours was allowed between tests under anesthesia, for an abnormal depression of the initial blood pressure has been sometimes observed with more frequent use of anesthesia. An interval of 24 hours was allowed between tests on control animals without anesthesia. These intervals seemingly had no effect on the results. The data given in Table II are indicative of the variation in response to be expected with a random selection of animals. In general, repeated tests on the same animal tend to be more consistent than tests on different dogs, but the results are still far from constant. There was no evidence that the use of an animal for assays over long periods of time affected the reliability of the results. For example, one dog was used for 18 tests in 8 weeks, and at no time showed evidence of the development of either a refractoriness or an increased sensitivity to the renin.

TABLE II.
Effect of Nembutal Anesthesia on the Blood Pressure Response of the Dog to Renin Injections.

		Unanesthetized				Anesthetized		
Dose per kg body weight γ	Dog	Initial blood pressure mm Hg	Peak blood pressure mm Hg	Rise in pressure mm Hg	Dog	Initial blood pressure mm Hg	Peak blood pressure mm Hg	Rise in blood pressure mm Hg
694*	1	96	175	79	1	94	257	163
		108	185	77		97	231	133
		100	185	85		102	229	127
		105	191	86		91	232	141
780*	2	113	215	102	2	130	191	61
		112	196	84		112	185	73
		122	206	84		116	215	99
		119	217	98		114	210	96
		112	199	87		109	204	95
50	1	100	170	70	1	99	145	46
		105	172	67		109	145	36
		106	168	62		110	153	43
		109	164	55		86	147	61
25	2	124	167	43	2	108	162	54
		120	151	31		102	143	41
		107	152	45		125	157	32
25	3	113	171	58	3	120	161	41
		126	172	46		131	165	34
		123	172	49		107	155	48
25	4	118	141	23	4	123	142	19
		118	148	30		129	154	25
		122	158	36		126	150	24

*1.5 cc extract (overdosage).

III. *Effect of Nembutal Anesthesia on the Response to Renin.* Five dogs in the previous series were given overdosages of renin both with and without anesthesia. Two of these (*e. g.,* Dog No. 1, Table II) showed significantly lower peak pressures and smaller rises when not anesthetized, a difference marked solely by a sudden interruption of the rise and a sustained holding of the pressure level at around 180 mm Hg in the unanesthetized tests. To check on this anesthesia difference, overdosages of the same extract were given 8 anesthetized and 7 unanesthetized dogs. The average blood pressure rises were 100 ± 3.4 and 70 ± 1.6, the peak pressures 211 ± 3.1 and 176 ± 2.3, respectively. This depression of the maximum peak pressure is present in enough dogs to make an assay of large doses of renin in the unanesthetized dog uncertain.

When smaller doses of renin were used, there was, in 2 dogs

(Nos. 1, 3, Table II) some indication of a depression of the rise with anesthesia. The other 3 showed no consistent difference between tests with and without anesthesia. Anesthesia has no definite effect on the variability of response in the dog.

For most routine testing, the anesthetized dog is the preferable test animal. If a sufficient time interval is allowed between tests, the repeated use of 2 or 3 dogs affords as accurate an assay as one based on 1 test each on a large series of dogs. Assays in both anesthetized and unanesthetized animals will show large variation, and a minimum of 10 tests is required for accuracy. Where the rise expected is clearly sub-maximal, the more rapid assay on unanesthetized dogs can be substituted.

Summary. The magnitude of the blood pressure rises given by large doses of renin must be corrected for differences in starting pressure levels, while those given by smaller doses need not be. An assay based on repeated tests on 2 or 3 dogs is as reliable as one based on a larger series of animals. Nembutal anesthesia may affect, in some dogs, the magnitude of the rise after large renin doses, but usually has little effect on the rise after small doses. For most routine testing, the anesthetized dog is the preferable test animal.

11721

Labyrinthine Disregard after Removal of the Caudate.*

FRED A. METTLER AND CECILIA C. METTLER.

From the Department of Anatomy, University of Georgia School of Medicine.

In an earlier research upon neostriatal function[1] the present authors observed that "It is difficult to escape the suspicion that animals (cats) with neostriatal injuries suffer from some variety of vestibular disturbance"; the literature relating to this observation was reviewed and it was noticed that Bergouignan and Verger[2] found that the ipsilateral circus movement (dogs) produced by unilateral caudate lesion was accentuated by rotation to the same side while rotation to the opposite side gave no circus movement. Cocainization of the labyrinth of either side always produced an accentuation of

---

* Financial assistance from the Milbank Memorial Fund is gratefully acknowledged.

[1] Mettler, Fred A., and Mettler, Cecilia C., *Role of the Neostriatum,* in press.

[2] Bergouignan, M., and Verger, P., *Compt. rend. soc. de Biol.,* 1935, **118**, 1539.

the original circus movement, never one toward the opposite side.[2] In the further work of Bergouignan and Verger there is, so far as we are aware, no conclusive evidence to indicate whether these results are due to disregard of the labyrinth of the same side or overemphasis of labyrinthine stimuli from the opposite side.[3] In order to arrive at some information on this point a series of animals were prepared in which (1) both frontal regions exclusive of the caudate nuclei were removed; (2) both frontal regions inclusive of the heads of the caudate nuclei were ablated, and (3) complete, bilateral, labyrinthine destruction had been carried out.

Rotation of animals of the first or second group produced effects of the same type as those seen in normal animals though there was less obvious distress exhibited by either type of operated animal during rotation than was displayed by normal cats. Further, although no part of the usual post-rotation symptomatology was absent or perverted, this did not persist so long in the operated as unoperated animals. These differences cannot be explained upon the basis of postoperative weakness, for the operated cats were in good health and had attained a stable state. Between the first and second group there were similar quantitative differences. No evidence of subjective discomfort whatever was exhibited by Group 2 during rotation (such as mewing) and, after rotation, the forced movements, postures and nystagmus subsided more promptly than in Group 1.

Following labyrinthine destruction, animals of the third group exhibited a crouching posture and dyskinesia especially in the form of ataxia accompanied by coarse tremors (more particularly when eating). Removal of the heads of both caudate nuclei, some weeks later, completely abolished these phenomena and except for the fact that such animals showed no post-rotatory effects they were quite indistinguishable from others, lacking only the caudate nuclei. This change from hesitating dyskinesia and a crouching, hypokinetic posture to full hyperkinetic, synergic locomotion, carried out with slightly increased extensor hypertonia and oversteppage was immediate.

The fact that the typical pattern associated with caudate damage ("leaping", hyperkinesia, resistance to impressed movements, etc.) immediately displaces the evidences of labyrinthine destruction demonstrates not only that these require the presence of either the frontal cortex or caudate or, as is more probable, both for their manifestation but also that all the phenomena of caudate injury may appear in the absence of the labyrinth and are thus not necessarily dependent upon labyrinthine stimulation.

---

[3] An article listed as Delmas-Marsalet, P., Bergouignan, M., and Verger, P., *Rev. de laryng.*, 1937, **58**, 353, has been inaccessible to us.

It is concluded (1) that, following removal of the heads of both caudate nuclei, labyrinthine stimulation exercises a reduced influence of a normal type upon the behavior of the animal and that (2) such removal abolishes the dyskinesia and hypokinesia, which follow destruction of both labyrinths, substituting in their place the typical effects which follow the former operation (namely "leaping," hyperkinesia, resistance to impressed movements and a certain degree of fatuity).

11722

Rate of Absorption of Amino Acids from the Small Intestine in Man.

Lemuel C. McGee and Edward S. Emery, Jr. (Introduced by A. Baird Hastings.)

From the Medical Clinic, Peter Bent Brigham Hospital, and the Departments of Medicine and Biological Chemistry, Harvard Medical School.

Using the Miller-Abbott[1] tube for intestinal intubation, observations were made on the disappearance rate of nitrogen from an amino acid mixture placed directly into the jejunum. Solutions of known concentration were introduced immediately above a single inflated bag at a point 50 to 80 cm below the pylorus, a method used by Groen[2] in studies on glucose absorption. Fasting jejunal juice was obtained before each series of observations and the figure found for nitrogen in each instance (0.6-1.2 mg cc) was subtracted from the results of analyses made on specimens withdrawn at intervals subsequent to the introduction of the solutions.

The subjects included 2 normal individuals, 2 with duodenal ulcer responding well to medical management, 3 with achlorhydria (well-controlled pernicious anemia patients), and one patient with a subtotal gastric resection. Repeated observations were possible with several subjects. As a control, the rate of absorption of unhydrolyzed protein was determined at least once for each of the 4 classifications mentioned above.

The amino acid mixture* used was "a purified casein digest prepared with pancreatic enzymes".[3] The amino acid powder dissolves readily in water warmed to 70° C. Solutions of from 2.5 to 5% were used. The disappearance rate of the nitrogen in such solutions was determined in 11 instances. (Chart 1.) Control observations were obtained by instilling unhydrolyzed casein dissolved in a solution of sodium carbonate and neutralized to a pH of 7.0 with hydrochloric acid (2 instances), a solution of gelatin in water (2 instances), and casein of milk (4 instances). (Chart 2.)

---

[1] Miller, T. G., and Abbott, W. O., *Am. J. M. Sci.*, 1934, **187**, 595.

[2] Groen, J., *New England J. M.*, 1938, **218**, 247.

* Product 92Z, Mead Johnson & Co.

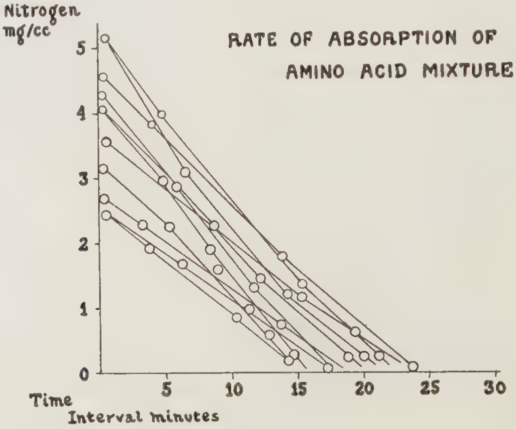


CHART 1.

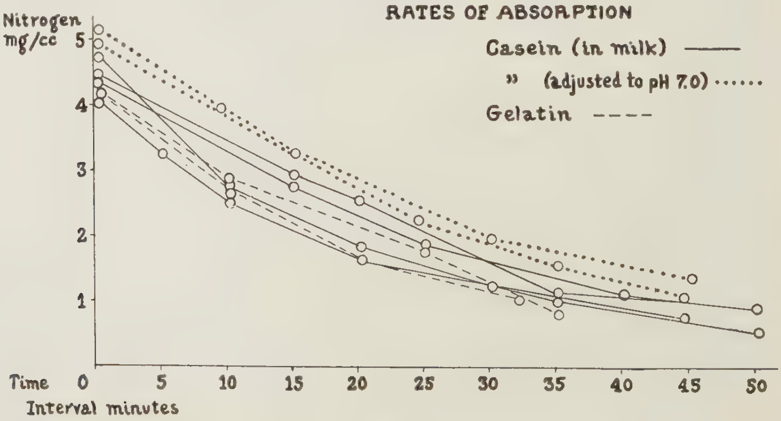


CHART 2.

Nitrogen determinations were made by the Kjeldahl method in the control group (using unhydrolyzed protein) and by both the Kjeldahl method and Sorensen (formol) titration when using solutions of amino acids.

---

[3] Cox, W. M., and Mueller, A. J., PROC. SOC. EXP. BIOL. AND MED., 1939, **42**, 658.

No essential differences in absorption of amino nitrogen from the jejunum were noted between the normal, the ulcer bearing, the pernicious anemia and the resection subjects. The results are summarized by plotting the milligrams of nitrogen per cubic centimeter in samples withdrawn through the tube against the time of withdrawal of the respective specimens.

Summary. (1) By means of intestinal intubation in man the absorption rate of hydrolyzed and unhydrolyzed protein from the jejunum has been estimated. (2) Most of the nitrogen of a 4 to 5% solution of casein and gelatin is absorbed 40 to 50 minutes after its introduction directly into the small intestine. (3) The nitrogen of an amino acid mixture (hydrolyzed casein) of similar concentration is fairly completely absorbed 15 to 25 minutes after its administration in the same manner.

11723

Failure to Infect the Great Horned Owl with the Sporozoites of *Plasmodium cathemerium.*

HARRY BECKMAN AND ROBERT K. OTA.

From the Department of Pharmacology, Marquette University School of Medicine, Milwaukee, Wisconsin.

Several years ago Wolfson[1] reported the intramuscular injection of a great horned owl with about 200 mg of blood from a canary which had a heavy mixed infection of *P. praecox* and *P. cathemerium.* Eight weeks after the injection of the owl a canary was injected intramuscularly with some of its blood and showed parasites in the peripheral blood after a prepatent period of about a week. Ten weeks after the injection of the owl a second canary was injected with some of its blood and showed parasites in the peripheral blood after a prepatent period of about 9 days. The first canary infection was predominantly *P. praecox* and the second predominantly *P. cathemerium.* The owl died at the tenth week and had never shown any organisms in the peripheral blood at any time. We have had the following experience in attempting a similar infection with sporozoites.

On January 30, 1940, two great horned owls (*Bubo virginianus virginianus*) were brought to our laboratory through the kindness of Dr. Malcolm Rogers of Milwaukee. One of the birds had been badly injured and we therefore made a smear of its peripheral blood at once and also withdrew some blood from the wing vein for injection into the canary, each of 6 canaries receiving intraperitoneally 0.25 cc of blood plus 0.05 cc of citrated saline solution. The owl died on the following day. The smears of its blood, Giemsa stained, were negative for plasmodia but positive for both *Haemoproteus* and *Leukocytozoon.* Five of the 6 canaries were examined on February 4th, 6th, 8th, 10th, 12th, 19th, 22nd, and 28th and did not show infection of any sort at any time; the sixth canary died on February 6th from causes unknown but had not shown any sign of infection up to that time.

---

[1] Wolfson, F., *Am. J. Hygiene,* 1937, **26**, 53.

The blood of the second of the horned owls contained no organism whatsoever on February 2nd at least so far as could be determined by thorough study of a Giemsa stained smear of its peripheral blood. On February 14th, at which time the blood was still negative, this bird was injected intramuscularly with a sporozoite mixture obtained by triturating the thoraces of 20 mosquitoes fed 10 days previously upon birds in the active stage of infection with *Plasmodium cathemerium,* in 0.4 cc saline solution and afterwards filtering over small glass beads. (The strain of *P. cathemerium* is a highly virulent one obtained from Dr. Clay G. Huff of the University of Chicago in February, 1937, and constantly passaged through *Culex pipiens* since that time; the method of sporozoite injection has yielded almost 100% of infections in our hands.). At the same time 2 clean canaries were also injected intramuscularly with a small amount of this sporozoite mixture.

The subsequent record is as follows. On February 22nd both of the canaries showed a high degree of infection with *P. cathemerium* and were subsequently used to infect clean mosquitoes. The peripheral blood of the owl was examined on February 19th, 23rd, 26th, March 1st, 5th, 9th, 14th, 19th, 25th, 28th, April 3rd, 6th, and 9th, the bird dying from an unknown cause on this day; none of these smears were positive, each being examined carefully for more than an hour. On each of the following days 2 birds were injected intraperitoneally with 0.15 cc of blood from the owl and 0.05 cc of citrated saline solution: February 14th (the day of the sporozoite injection), March 19th, March 27th, and April 3rd (7 weeks after the sporozoite injection); no positive blood smears were ever obtained from any of these birds and all of them have subsequently become infected by sporozoite injection in the routine manner. On the day of the owl's death groups of 2 canaries each were injected with saline triturations of bone marrow, spleen, brain, and liver respectively; none of these birds became infected, though the spleen and liver injected birds died from shock too soon for the malarial infection to have developed.

Summary. We have reported here the failure to infect a great horned owl (*Bubo virginianus virginianus*) with the sporozoites of a strain of *Plasmodium cathemerium* of known infectivity and virulence.

11724

Effect of Ascorbic Acid on Guinea Pig Colon.

Harvey B. Haag and Isabel Taliaferro.

From the Department of Pharmacology, Medical College of Virginia, Richmond.

Prompted by clinical reports[1-3] that the administration of ascorbic acid occasionally gives rise to intestinal colic and to an increase in intestinal motility, we have observed the effect of this drug on the isolated guinea pig colon.

Experimental. The colon was removed, and kept in Locke's solution as modified by Sollmann and Rademaekers[4] except that the $NaHCO_3$ was 0.1% to 0.2%, depending upon the pH desired (*vide infra*).

The sections were about 2 cm long and were ligated at each end, with the two knots in line in relation to the circumference of the strip. Recordings were made after the so-called Magnus method in a bath of 50 cc, with the temperature constant at 38° C. Before filling the bath the stock Locke's solution was brought to 38° C, and bubbled with a gas mixture consisting of 95% O_2 and 5% CO_2 to the desired pH as determined with the glass electrode pH meter. The bath was then filled and thereafter bubbled at a constant rate with the gas mixture through a capillary tube. The electrodes were placed in the bath and pH readings were made at frequent intervals. This technic ensured thorough oxygenation, mixing, and a constant pH which could be read at any given moment. Washings from the bath were analyzed for potassium content. These showed no increase in potassium, demonstrating the absence of leakage from the calomel electrode.

---

[1] Anderson, S., personal communication.

[2] Schade, H. A., *Klin. Wchnschr.*, 1935, **14**, 60.

[3] Widenbauer, F., *Klin. Wchnschr.*, 1936, **15**, 1158.

[4] Sollmann, T., and Rademaekers, A., *Arch. Internat. de Pharmacodyn. et de Therap.*, 1926, **31**, 39.

Ascorbic acid* was titrated with NaOH to a pH of about 7.4 (phenol red) and the solution adjusted so that the volume of the test dose was always 0.5 cc. After a normal tracing had been obtained for about 15 minutes, the freshly prepared solution was slowly added to the bath. Between different tests on the same strip the bath was usually renewed once or twice. The preparation remained uninjured with the concentrations of ascorbic acid (up to 10 mg%) used, and on return to normal was again responsive to the drug.

Twenty-three experiments were performed on 11 guinea pigs. In 7 of these the Locke's solution contained 0.2% $NaHCO_3$ and the pH of the bath among individual experiments varied from 7.78 to 7.83. In the other 16 the bicarbonate content was from 0.10% to 0.12% and the pH of the bath was from 7.37 to 7.53. With care individual experiments could be run for 2 hours or more with the pH constant to 0.03 of a pH, the readings being taken every 3 to 5 minutes. In no case did the addition of the sodium salt of ascorbic acid change the pH of the bath. Although the guinea pig ileum has been said to be particularly sensitive to pH changes,[5] this is apparently not true of the colon since we have often noticed that a change of as much as 0.1 pH had no effect on the normal contractions.

Results and Comments. The results of these experiments, a typical

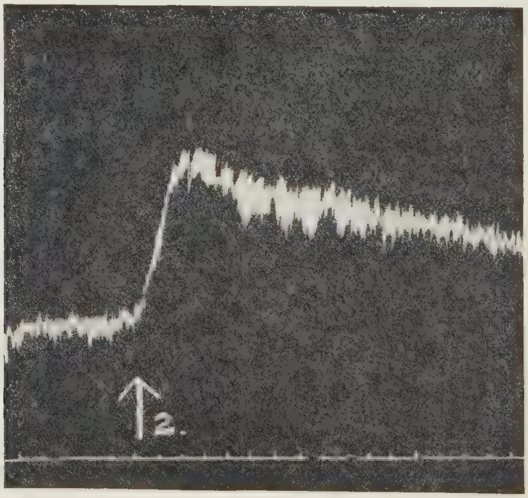


FIG. 1.

Showing the effect of ascorbic acid, 10 mg% (↑2), on the isolated guinea pig colon. Time 1 minute.

---

* "Cebione"—purchased from Merck & Co.

[5] Code, C. F., *J. Physiol.*, 1937, **89**, 257.

tracing from which is given in Fig. 1, show that:

1. The tone of the excised guinea pig colon was greatly increased by the addition of the sodium salt of ascorbic acid to the constantly oxygenated Locke's bath.
2. This effect was obtained with reagent quality chemicals and Pyrex redistilled water with or without the addition of a trace of copper. Such addition of copper (.1-4.0 micro-mols per liter of copper ion, as chloride) was suggested by Peugnet[6] for obtaining positive results with ascorbic acid on the isolated frog heart.
3. The effect has been obtained uniformly at pH values ranging from 7.37 to 7.83. Observations at other pH figures were not made.
4. The increase in tone began in from 1 to 2 minutes and reached its maximum in from 2 to 5 minutes. Thereafter the tone gradually decreased until it reached normal.
5. The test concentration used in most cases was 10 mg% ascorbic acid. The threshold concentration (6 strips from 4 guinea pigs) was from 1 to 5 mg%. These latter figures approach those reported for human blood plasma.[7, 8]

Similar studies, but with less consistent and less striking results were made on the isolated jejunum of the guinea pig and rabbit. Kreitmair[9] obtained no effect on the rabbit intestine (small) with a concentration of ascorbic acid of 1-100,000; on a similar preparation Supniewski and Hano[10] obtained positive results with a concentration of 1-10,000 and on the intestines of the anesthetized cat following the intravenous injection of 100 mg (per kg?) of the chemical. Nishimura[11] briefly reported somewhat equivocal results on the isolated rabbit intestine.

Summary. Ascorbic acid, in the concentrations studied (up to 10 mg%), markedly increased the tone of the isolated guinea pig colon. This experimental observation is in keeping with the clinical impression that ascorbic acid administration may occasionally produce colicky pains of intestinal origin.

---

[6] Peugnet, H. S., *Science*, 1939, **90**, (2329) 162.

[7] Stephens, D. J., and Hawley, E. E., *J. Biol. Chem.*, 1936, **115**, 653.

[8] Abt, A. F., Farmer, C. J., and Epstein, I. M., *J. Ped.*, 1936, **8**, 1.

[9] Kreitmair, H., *Arch. f. exp. Path. u. Pharm.*, 1934, **176**, 326.

[10] Supniewski, J. V., and Hano, J., *Arch. f. exp. Path. u. Pharm.*, 1935, **178**, 508.

[11] Nishimura T., *Mitt. u. d. Med. Akad. zu Kioto*, 1937, **21**, 423.

11725

Curative Effect of Pantothenic Acid on Adrenal Necrosis.*

R. C. MILLS, J. H. SHAW, C. A. ELVEHJEM AND P. H. PHILLIPS.
From the Department of Biochemistry, College of Agriculture, University of Wisconsin, Madison.

During the past 2 years we have frequently observed adrenal damage in rats kept on purified rations similar to J29.[1] The glands exhibit a definite pinkish or even purple color and in severe cases are greatly enlarged. Histological examination of these glands showed the condition to be an hemorrhagic cortical necrosis similar to that reported by Daft and Sebrell[2] and described by Nelson.[3] These workers stated that the condition was due to a deficiency of a dietary factor present in the fuller's earth filtrate of liver extract. We found that several different liver extract fractions would prevent the condition, and since some of these fractions were low in pantothenic acid we felt that the active factor might be separate from this vitamin. When synthetic pantothenic acid became available we were able to study the active agent more thoroughly.

The basal ration used (J36) consisted of sucrose 76%, alcohol-extracted casein 18%, salts III[4] 4%, corn oil 2%, thiamin chloride, vitamin B_6, and riboflavin 300γ per 100 g of ration, nicotinic acid 2.5 mg per 100 g, and choline hydrochloride 30 mg per 100 g. Two drops of haliver oil were fed by dropper each week. Rats were placed on experiment at 3 weeks of age.

In several different series the adrenals from rats on the basal ration were compared with those from rats receiving 25, 50, or 100 γ of calcium pantothenate per day. In other groups similar levels of pantothenic acid were fed, but with food intake restricted to that of rats receiving no pantothenic acid. Additional choline and a liver extract preparation were also fed. The rats were killed by decapitation, usually after being on experiment for 4 to 6 weeks, and the adrenals prepared immediately for histological study.

---

* Published with the approval of the Director of the Wisconsin Agricultural Experiment Station. This work was supported in part by grants from the Research Fund of the University.

We are indebted to Merck and Company, Rahway, New Jersey, for supplies of thiamin, nicotinic acid, vitamin B_6, calcium pantothenate, and to Abbott Laboratories, North Chicago, Illinois, for haliver oil.

[1] Oleson, J. J., Elvehjem, C. A., and Hart, E. B., PROC. SOC. EXP. BIOL. AND MED., 1940, **43**, 161.

[2] Daft, F. S., and Sebrell, W. H., *Pub. Health Reports*, 1939, **54**, No. 51, 2247.

[3] Nelson, A. A., *Pub. Health Reports*, 1939, **54**, No. 51, 2250.

[4] McKibbin, J. M., Madden, R. J., Black, S., and Elvehjem, C. A., *Am. J. Physiol.*, 1939, **128**, 102.

The results of the experiments are summarized in Table I. Of the 15 rats receiving the basal ration alone, 3 had normal adrenals, 3 had adrenals showing hemorrhage or early necrosis, and 9 had adrenals which were severely necrotic. One of the normal cases was killed after only 2 weeks, so that the condition may not have had time to develop. All the others were killed between 4 and 6 weeks.

TABLE I.

Diet	Total No. of rats	Avg weekly gain g	Adrenals		
			Normal	Early necrosis	Advanced necrosis
J36 only	15	5.8	3	3	9
J36 (with choline at 200 mg/100 g)	7	8.5	1	0	6
J36 + calcium pantothenate at					
25 γ/day	3	9.6	3	0	0
50 ”	10	15.8	10	0	0
100 ”	13	15.6	13	0	0
As above with restricted intake	6	8.5	6	0	0
J36 + factor W concentrate = 0.2 g L.E.	7	18.8	7	0	0

When calcium pantothenate was given at levels of 25, 50, or 100 γ per day the necrosis was completely prevented in all 32 cases. The food intake of 6 of these rats was restricted to that of rats on the basal ration alone, so that increased consumption could not supply more of any other factor to prevent the necrosis. Three of the 6 rats received 50 γ, and the other 3, 100 γ per day; the adrenals of all were normal.

In order to eliminate the possibility of the condition being a choline deficiency, 7 rats received the basal ration in which the choline level had been increased from 30 to 200 mg per 100 g. Of these 7 rats, 6 showed very severe adrenal necrosis at 4 and 6 weeks; the seventh had normal adrenals, but was killed after only 2 weeks. Choline seemed to aggravate rather than prevent the condition.

Liver extract which had been autoclaved in 1N sulfuric acid for 1 hour was fed to 7 rats at a level of 0.2 g per day. The adrenals of the rats were normal. Although the autoclaving destroyed most of the pantothenic acid in the liver extract, enough remained so that the rats received about 4 γ per day. This amount was evidently sufficient to prevent the occurrence of adrenal necrosis on our ration.

Summary. Rats receiving only our basal ration J36 showed an hemorrhagic cortical necrosis of the adrenals after 4-6 weeks. This condition was prevented by calcium pantothenate. Choline did not prevent but rather aggravated the condition. Acid autoclaved liver extract prevented the condition, but its action may have been due to its pantothenic acid content.

The activity of pantothenic acid in the prevention of adrenal necrosis has been reported by Daft, F. S., Sebrell, W. H., Babcock, S. H. J., and Jukes, T. H., (*Public Health Rep.*, 1940, **55**, 1333) but due to an oversight the authors did not find their paper until after this note was submitted for publication.

11726 P

Effect of Testosterone Propionate on Regenerating Anal Fin of Adult *Platypoecilus maculatus* Females.*

CLIFFORD GROBSTEIN. (Introduced by B. M. Allen.)

From the Department of Zoology, University of California, Los Angeles.†

It has been found previously that administration of testosterone propionate to adult female poeciliid fishes masculinizes the anal fin but does not produce a typical male copulatory organ or gonopod (Regnier[1]—*Xiphophorus helleri;* Grobstein[2]—*P. maculatus, P. variatus*). The present experiments were performed to determine whether the incomplete masculinization may be due, in whole or in part, to lack of capacity of the reacting fin for gonopod formation.

Sixty-six adult *P. maculatus* females (9 months of age or older, 25 to 35 mm in length) were injected intraperitoneally with testosterone propionate during anal fin regeneration. In the 5 separate series that were run, both dosage and the interval between time of removal of the anal fin and beginning of injection were varied in an effort to obtain the most typically male response possible. Concentrations of 2.5, 5.0 and 25.0 mg/cc were used and injection was begun at intervals of 0, 5, 10, 12, 20, 24 and 56 days after removal of the anal fin. In all series but one 0.05 cc of solution was injected every fifth to seventh day until the response was completed.

Controls were of 3 types: 15 were untreated during anal fin regeneration, 10 were injected during regeneration with sesame oil.

---

* These data have been included in a thesis submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, University of California, Los Angeles, June, 1940. I am indebted to Dr. A. W. Bellamy for materials and facilities. Testosterone propionate (Oreton) was generously supplied by Dr. Erwin Schwenk of Schering Corp.

† Now in the Department of Zoology, Oregon State College, Corvallis, Oregon.

[1] Regnier, M., *Bull. Biol.*, 1938, **72**, 385.

[2] Grobstein, C., unpublished data.

the medium in which the hormone was administered, and 17 were normal females receiving testosterone propionate of appropriate concentration simultaneously with the experimental animals.

The anal fin was removed with sharp surgical scissors by cutting across the rays approximately one millimeter from the body. In all cases good ray stumps remained. Bleeding was absent or limited. Mortality, either from injection or anal fin removal, was negligible.

Results. Following amputation a characteristic female anal fin is regenerated by normal or sesame-oil-injected females in approximately 30 to 40 days. Under the influence of testosterone propionate, however, a structure is produced (Figure 1-C) which, while not typical, is clearly more like the normal male gonopod[3] (Figure 1-A) than that produced by treating females with intact anal fins (Figure 1-B). The third ray of the masculinized regenerate (Figure 1-C, 3) is strongly thickened and, together with the fourth and fifth rays, is elongated to form a short, but conspicuous promontory ("3, 4, 5 complex"). Within the promontory may be recognized the various specialized structures characteristic of the normal gonopod, *viz.*, spoon support (ss), spoon (sp), proximal teeth (pt4), distal teeth (dt), terminal hook (th), and blade (bl). The specialized structures

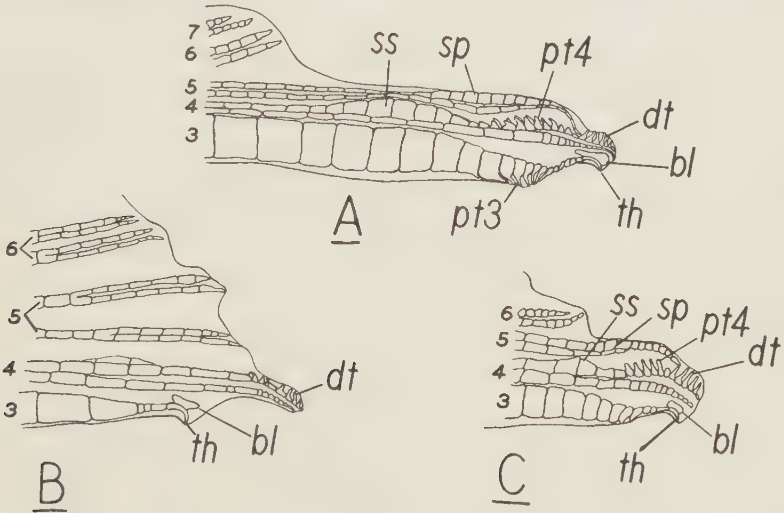


FIG. 1.

A. Distal half of normal male gonopod. B. Distal portion of rays 3, 4, 5, 6 of masculinized anal fin from a normal adult female injected with testosterone propionate. C. Distal portion of rays 3, 4, 5, 6 of anal fin regenerated by an adult female injected with testosterone propionate. bl—blade; dt—distal teeth; pt3—proximal teeth of third ray; pt4—proximal teeth of fourth ray; sp—spoon; ss—spoon support; th—terminal hook.

---

[3] Grobstein, C., *Univ. Calif. Publ. Zool.*, 1940, **47**, 1.

resemble those of the normal male but are more or less atypical in form. Thus, the teeth and terminal hook are thick and blunt, the blade is poorly shaped, the spoon is a jumbled mass, and the spoon support is little more than a thickening of 2 or 3 segments in the caudal ramus of the fourth ray.

While some variation in structural detail has been observed with the different concentrations of hormone injected, the fundamental pattern of the response appears to be the same. Greater differences were obtained by varying the interval between the time of removal of the anal fin and the beginning of injection. When injection was begun from 0-20 days after removal of the anal fin the response was essentially similar to that described above. Beyond 20 days, however, there was a noticeable decline in the capacity for gonopod formation and, by 56 days, the structure produced was very much like that resulting from injection of normal adult females (Figure 1-B).

The data so far available support the idea that the anal fin during the regenerative course passes through a period of maximal capacity for gonopod formation and that this capacity declines as the definitive female form is attained. It is likely, therefore, that at least one factor involved in the incomplete and atypical masculinization of the intact adult female fin by testosterone propionate is limited capacity of the reacting tissues for gonopod formation.

Summary. Treatment of adult *P. maculatus* females with testosterone propionate, begun during the first 20 days of anal fin regeneration, produces an anal fin which is more male-like than that obtained by treatment of normal females but which differs clearly from the typical male gonopod. Beyond 20 days of regeneration the capacity for gonopod formation declines until, by the time the characteristic female form is attained, the capacity is no greater than in the normal adult female fin.

11727 P

Choline and Epithelial Hyperplasia in the Forestomach of Rats.

George R. Sharpless. (Introduced by F. W. Hartman.)

From the Department of Laboratories, Henry Ford Hospital, Detroit, Mich.

In a recent study[1] of rats fed a white flour diet, supplements of cystine, lactoflavine, nicotinic acid, and rice polish concentrate prevented hyperplasia of the forestomach epithelium. Experiments with known factors of the rice polish concentrate have shown that choline is essential for prevention of lesions. The basal diet was composed of white flour 89.0%, salts 5.0%, butterfat 5.0%, nicotinic acid 0.5%, cystine 0.5%, riboflavin 0.4 mg %, thiamin hydrochloride 1.0 mg %, and pyridoxine hydrochloride* 2.0 mg %. Young rats weighing between 50 and 70 g were fed this diet for six weeks. They were then killed and their stomachs were examined for the papillomatous lesions that have been described.[1]

Eighteen of a group of 26 rats fed the basal diet had lesions. The changes varied from one small papillomatous area near the gastric orifice of the esophagus to a large number of individual areas distributed over the whole inner surface of the forestomach. Thirty-three rats, litter mates of those fed the basal diet, were fed the same diet supplemented by 0.15% choline hydrochloride. Only 5 of the 33 had lesions. Four of these had one and the fifth had only 2 small papillomatous areas. Choline was the only variable, which leads to the conclusion that it is one of the factors necessary to reduce the incidence of hyperplasia of epithelium in the forestomach of rats fed white flour.

The relation of choline to prevention of fatty livers is well known. More recently hemorrhagic degeneration of the kidneys has been obtained in young rats fed choline deficient diets[2] and Du Vigneaud[3] has shown that homocystine will substitute for methionine when choline is present. Demonstration of the essential nature of choline in prevention of epithelial hyperplasia is further evidence of the value of choline or substances with choline-like action in the rats' diet.

While the fundamental cause of the stomach changes remains obscure, proof that choline helps to maintain normal epithelium gives a basis for correlation of two previous observations that had no apparent common relationship, namely protein tends to prevent and fat[5] tends to aggravate the lesions. In fact, Hoelzel and Da Costa[4] have maintained that the changes are produced by a protein deficiency. Since methionine and perhaps other amino acids have a choline-like action, protein would be expected to help prevent the lesions. Because choline is necessary for normal fat metabolism or transport a choline deficiency would tend to follow ingestion of a high fat diet.

---

[1] Sharpless, George R., *J. Nutrition*, 1940, **19**, 31.

* The pyridoxine hydrochloride was kindly furnished by Merck and Co., Inc.

[2] Griffith, Wendell H., and Wade, Nelson J., *J. Biol. Chem.*, 1940, **131**, 567.

[3] Du Vigneaud, Vincent, Chandler, Joseph P., Moyer, A. W., and Keppel, Dorothy M., *J. Biol. Chem.*, 1940, **131**, 57.

Summary. Choline is necessary to help maintain normal squamous epithelium in the forestomach of rats fed white flour. This observation explains why protein tends to prevent and fat tends to increase hyperplasia of the forestomach epithelium.

11728

Salt after Adrenalectomy. III. Carbohydrate Stores in Adrenalectomized Rats Given Various Levels of Sodium Chloride.*

Evelyn Anderson, Virgil Herring and Michael Joseph.
(Introduced by Herbert M. Evans.)

From the Institute of Experimental Biology and the Department of Medicine, University of California, Berkeley and San Francisco, California.

In a preceding communication[1] it was shown that giving 1% sodium chloride solution to adrenalectomized rats enabled them to grow and maintain a satisfactory state of health, and on the other hand, a larger amount of sodium chloride proved to be deleterious. The capacity of the adrenalectomized rats to store fed glucose has been studied under these 2 types of treatment. A group of 35 adrenalectomized rats and 21 "sham adrenalectomized" control rats were allowed to drink 1% sodium chloride solution in place of tap water. The average NaCl intake was 940 mg daily. Another group of 5 adrenalectomized rats and 4 controls were given tap water to drink. The NaCl intake was 339 mg daily for the former and 601 mg for the latter. The rats were kept in individual cages in a constant temper-

---

[4] Hoelzel, Frederick, and Da Costa, Esther, *Am. J. Digest. Dis. and Nutrition*, 1937, **4**, 325.

[5] Fujimaki, Yoshitomo, *Trans. Jap. Path. Soc.*, 1931, **21**, 708.

* We wish to acknowledge the assistance of the Federal Works Progress Administration, Project No. OP 65-1-08-62, Unit A-5, and the Christine Breon Fund.

[1] Anderson, E., Joseph, M., and Herring, V., Proc. Soc. Exp. Biol. and Med., 1940, **44**, 477.

ature chamber at 28.5° C. The food and fluid intake and the gain in body weight were carefully measured. These data are given in Chart II of the previous communication.[1] The methods used and the standardization of the conditions necessary for this experiment have been described previously.[2]

Another group of 8 adrenalectomized rats and 8 controls on 1% NaCl were followed for 176 days. These animals were tested for completeness of adrenalectomy by withdrawing salt and allowing them to go into a state of adrenal insufficiency. This appeared within 24 hours. A striking decrease in food intake occurred and the loss in weight averaged 10 g daily. It was not safe to permit the animals to continue without salt more than 48 hours. At autopsy a block of tissue was removed from each adrenal site and serial sections made. No evidence of adrenal tissue was found. This group of animals continued to grow during the entire period of the experiment. Fig. 1 shows the gain in body weight of these animals compared with the gain in weight of the control rats. The gain in weight is practically the same for both groups.

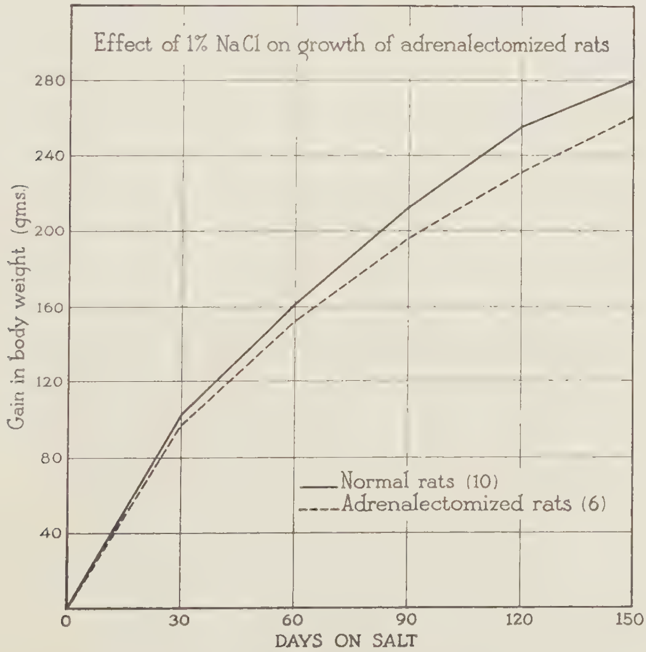


Fig. 1.

Showing the Gain in Body Weight of Adrenalectomized Rats Given 1% NaCl.

---

[2] Anderson, E., Joseph, M., and Herring, V., Proc. Soc. Exp. Biol. and Med., 1939, **42**, 785.

TABLE I.
Effect of 1% NaCl on Carbohydrate Stores of Adrenalectomized Rats.

	No. animals	Glucose fed, g	Intestinal absorption %	Blood sugar mg %	Muscle Glycogen mg %	Liver glycogen mg %
Group I 15 days post-oper.						
No NaCl soln. adr.*	5	1.2	73.3 ± 6.8‡	133 ± 15.6	535 ± 44.3	432 ± 224
contr.†	4	1.2	91.3 ± 5.1	138 ± 6.2	558 ± 54.8	1,977 ± 264
Group II, 10 days post-oper.						
1% NaCl adr.	10	1.2	92.7 ± 2.0	133 ± 6.2	606 ± 53.4	1,494 ± 54.1
control	6	1.2	95.5 ± 1.1	138 ± 5.8	712 ± 19.3	1,760 ± 266.6
Group III 15 days post-oper.						
1% NaCl adr.	7	1.2	88.1 ± 3.6	137 ± 12.7	543 ± 30.5	1,074 ± 178.4
control	4	1.2	97.2 ± 1.0	128 ± 17.9	760 ± 73.6	2,150 ± 337.2
Group IV 20 days post-oper.						
1% NaCl adr.	7	1.2	93.8 ± 3.5	143 ± 6.7	589 ± 35	1,056 ± 189.1
control	4	1.2	98.5 ± 1.3	155 ± 5.0	800 ± 128	1,711 ± 427
Group V 50 days post-oper.						
1% NaCl adr.	4	1.2	93.2 ± 4.5	147 ± 20.9	—	1,412 ± 160
control	3	1.2	98.0 ± 1.0	195 ± 28.8	—	1,933 ± 202
Group VI 176 days post-oper.						
adr.	8	2.5	89.6 ± 2.2	123 ± 11.4	—	1,233 ± 175
control	8	2.5	73.3 ± 3.2	177 ± 27.6	—	1,886 ± 145

*Adrenalectomized.
†Sham adrenalectomized.
‡Standard errors used throughout this table.

TABLE II.

Effect of High NaCl Intake in Adrenalectomized Rats upon Carbohydrate Stores.

	No. animals	NaCl daily g	Intestinal absorption %	Blood sugar mg %	Muscle Glycogen mg %	Liver glycogen mg %
Adrenalectomized rats 15 days post-oper.						
5% NaCl adr.	7	0.8†	86.7 ± 1.4	155 ± 16.5	124 ± 28.7	630 ± 159.1
5% NaCl control*	4	0.8†	95.7 ± 1.5	141 ± 4.9	435 ± 70.8	1,202 ± 292
1% NaCl adr.	7	0.32‡	88.1 ± 3.6	137 ± 12.7	543 ± 30.5	1,074 ± 178.4
1% NaCl control	4	0.41‡	97.2 ± 1.0	128 ± 17.9	760 ± 73.6	2,150 ± 337.2
Adrenalectomized rats 20 days post-oper.						
5% NaCl adr.	6	0.8†	89.3 ± 3.0	126 ± 7.9	415 ± 60.7	676 ± 87.9
5% NaCl control	3	0.8†	94.0 ± 4.4	137 ± 15.9	423 ± 52.8	1,433 ± 74.5
1% NaCl adr.	7	0.34‡	93.8 ± 3.5	143 ± 6.7	589 ± 35	1,056 ± 189.1
1% NaCl control	4	0.49‡	98.5 ± 1.3	155 ± 0.5	800 ± 128	1,711 ± 427

*Control rats subjected to a sham adrenalectomy.

†Amount of NaCl given by stomach tube.

‡Amount of NaCl taken voluntarily in 1% solution.

Standard errors have been used throughout this table.

The rats were sacrificed on the 10th, 15th, 20th, 50th and 176th days post-adrenalectomy. The animals were fasted for 20 hours, and then fed a standard glucose meal (5 cc of 25% solution=1.2 g glucose to all the animals except those of Group VI which received 10 cc of 25%=2.5 g glucose) and 4 hours later anaesthetized and the blood and tissues taken for the following determinations: intestinal absorption of glucose, blood sugar, liver glycogen and muscle glycogen. The results are given in Table I. It will be seen from this table that 1% NaCl enables the adrenalectomized rat to absorb fed glucose and to store liver glycogen almost as well as its corresponding control animal. The value for liver glycogen remains high in all the groups through 176 days. The levels of blood sugar were not significantly different in the experimental and control groups over the entire period of 176 days. The muscle glycogen values were unchanged over the period in which they were determined.

In another experiment the effect of high NaCl intake on carbohydrate storage was studied and compared with the effect of 1% NaCl. Thirteen adrenalectomized rats and 7 control rats subjected to a "sham adrenalectomy" were given 4 cc of a 5% NaCl solution 4 times a day. The food, fluid and salt intake on this set of animals has been reported in the preceding communication.[1] The total NaCl intake was 1234 mg daily for the adrenalectomized rats and 1352 mg daily for the controls. The animals were sacrificed on the 15th and 20th days post operative. Similar determinations were made as for the groups described above. The data are given in Table II. On inspection of these data, it is apparent that with the higher NaCl intake there is an impairment in the storage of liver glycogen from fed carbohydrate by the 15th post operative day and this is further substantiated by the findings on the 20th day after adrenalectomy. Furthermore, the high NaCl intake seemed to prevent the high glycogen storage seen in intact animals on 1% NaCl.

Long, Katzin and Fry[3] state that so long as adrenalectomized rats are fed, normal levels of liver and muscle glycogen are found. This appears to be true after adrenalectomy provided a certain optimal amount of NaCl is given. For instance, the daily intake of 650 to 940 mg NaCl enables the adrenalectomized rat to store fed glucose almost as well as the normal animal for 176 days post operative but adrenalectomized rats kept on NaCl levels below or above this optimal range are not able to store fed glucose as normal animals. For instance, the adrenalectomized rats of Group I, Table I given tap water to drink had a marked reduction in liver glycogen by the 15th day post operative.

---

[3] Long, C. N. H., Katzin, B., and Fry, E., *Endocrinology*, 1940, **26**, 309.

Summary. The adrenalectomized rat given 650-940 mg NaC1 daily stores fed glucose as liver glycogen almost as well as does an intact animal. A NaC1 intake of 1200 mg seems to interfere with glycogen storage in both adrenalectomized and intact animals.

11729 P

Electrical Conveyance of the Melanophore Hormone.

J. C. MUSSIO FOURNIER, O. CONTI AND J. C. LABORDE.
(Introduced by G. W. Corner.)

From the Institute of Endocrinology, Montevideo, Uruguay.

The treatment of vitiligo[1] with the melanophore hormone as developed in this Institute has all the disadvantages peculiar to intradermal injections. Electrical conveyance has now been explored as a means of facilitating therapeutic application. Experiments have been carried out along 2 lines, *i. e.*, conveyance of the hormone *in vitro* and conveyance through the skin.

1. *Conveyance in vitro.* The apparatus employed (Fig. 1) consists of a series of 7 vessels joined together by means of siphons filled with saline. The solution containing the melanophore hormone, which has been purified by a modification of Stehle's method,[2] is placed into the central vessel. The distribution of the hormone in the system was ascertained by biological test after 1 mA current had passed through the apparatus for 24 hours.

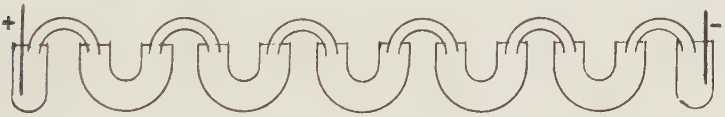


FIG. 1.

Dietel's method[3] of testing, adapted to the native species *Leptodactylus ocellatus,* was employed. In all the anodic vessels the test was negative, whereas decreasing amounts of melanophore hormone were found in the 2 vessels nearest to the central one. Saline solution was used in the experiments described here, which were preliminary, because we lacked the necessary potential for experiments with distilled water. However, we are now engaged with electrophoresis studies, in which we use the latter medium, following Williams'[4] technique. Our aim is to corroborate the findings obtained by the saline method, and to compare, if possible, the speed at which the melanophore hormone is conveyed with the conveyance of the hormones of the posterior lobe, the oxytocic and vasopressor factors, for which data are available from the work of du Vigneaud, *et al.*[5]

---

[1] Mussio Fournier, J. C., Cervino, J. M., and Conti, O., *Bull. de l'Acad. de Med.*, 1938, **120**, 770; Mussio Fournier, J. C., Cervino, J. M., and Conti, O., in press.

[2] Stehle, R. L., *J. Pharm. and Exp. Therap.*, 1936, **57**, 1.

[3] Dietel, F. G., *Klin. Wochenschr.*, 1932, **11**, 2075.

2. *Conveyance Through the Skin.* In order to study the passage of the melanophore hormone through the skin, 2 frogs were put in series in a circuit of galvanic current. A cotton disc 25 mm in diameter and 5 mm thick was applied over the median-dorsal area of each frog, and 2 carbon electrodes were pressed down upon the discs. After saturating both discs with the solution containing the melanophore hormone (about 100 frogs units per cc), the circuit is switched on and a 1 mA current is allowed to pass for 15 minutes. At the end of this time the frog bearing the positive electrode showed a distinct brown spot exactly reproducing the form of the electrode applied on that part (as was to be expected, since the hormone goes through the skin at the place where the positive terminal is applied) while no

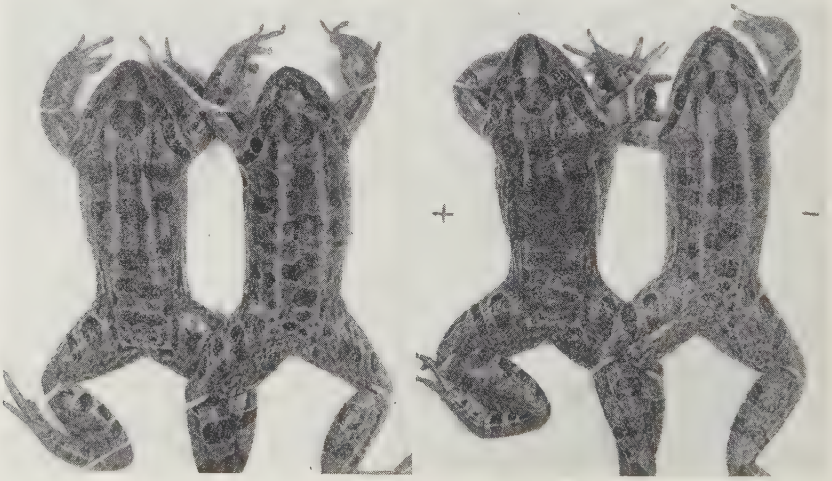


FIG. 2.

---

[4] Williams, J., and Truesdail, J. H., *J. Am. Chem. Soc.*, 1931, **53**, 4171; Williams, R. J., Lyman, C. M., Goodyear, J. H., Truesdail, J. H., and Holaday, D., *J. Am. Chem. Soc.*, 1933, **55**, 2912; Williams, R. J., and Moser, R., *J. Am. Chem. Soc.*, 1934, **56**, 169; Williams, R. J., *J. Biol. Chem.*, 1935, **110**, 589.

[5] du Vigneaud, Vincent, Irving, G. W., Dyer, H. M., and Sealock, R. R., *J. Biol. Chem.*, 1938, **123**, 45; Irving, G. W., and du Vigneaud, V., *J. Biol. Chem.*, 1938, **123**, 485.

change at all was visible on the skin of the other frog. The brown spot persists for a few hours and then slowly disappears, while a slight darkening of the whole skin becomes noticeable.

Figure 2 demonstrates this darkening process. The first photograph shows the frogs, both of the same colour and characteristics, before the passage of the current; on the second photograph the darkening at the place where the positive electrode was applied is clearly visible.

This experiment was repeated many times with the same results.

All control experiments carried out by saturating the electrodes with distilled water or saline solution were negative. Neither does application of the hormone solution to the skin, without passage of the current, produce any change.

11730

A Lethal Dermatitis in Chickens Produced by External Application of Fat.

Ben-Ami Ben-Dor. (Introduced by H. J. Almquist.)

From the Division of Poultry Husbandry, College of Agriculture, University of California, Berkeley.

It was noticed during the course of a joint experiment of the Institute of Experimental Biology and the Poultry Division of the University of California that Single Comb White Leghorn chicks fed purified diets containing 40% fat contracted a severe dermatitis. This dermatitis was found not to be of nutritional origin, since chicks on a normal diet whose entire skin was coated with the same fat developed the same dermatitis and died within 3 to 5 days. One or two applications were sufficient in the case of chicks 4-5 weeks old; older birds were more resistant. Furthermore, the ingestion of the purified diets containing 40% fat was shown to be harmless if care was taken to prevent the fat from coming in contact with the superficial skin.

Thirty-one chicks were used in the following experiment. They were kept in battery brooders provided with wire mesh floors, and fed the normal chick mash. The 6 control chicks remained in perfect health. The coating was performed by gently applying the oil or liquefied fat (heated to about 42° C) to the skin of the chick by means of a cotton swab, or by pouring it between the feathers di-

TABLE I.
Effect of Coating Chicks' Entire Skin with Fat.

No. of chicks	Age in wk	Fat or oil used	Times treated	Reaction of chicks*
6	4	Lard	1	All died within 4 days
1	5	,,	1	Healed
1	5	,,	2	,,
1	7	,,	1	,,
1	7	,,	6	,,
1	7	Cottonseed oil	1	Died within 5 days
1	7	,, ,,	3	Healed
1	7	Hydrogenated cottonseed oil	5	,,
1	7	Chicken fat	1	Died within 5 days
2	7	Mineral oil	1	One died within 3 and the other within 5 days

*Coating the skin with fat invariably caused dermatitis.

rectly onto the skin. The results of such treatment are given in Table I.

Coating a limited area of the body produced a dermatitis limited to that area. The chick in this case survived the treatment. The following treatments, applied to chicks of the same age, failed to produce the dermatitis:

1. Injection of fresh or rancid fat (lard) in amounts which, when applied externally, caused dermatitis and death.
2. Subjection of the skin of the chick's entire body (with the exclusion of the head) to an atmosphere of CO_2 for a period of 7 days.

Gross lesions and histological findings: In the last stages of the dermatitis the chick was listless; the skin was hyperaemic, it ruptured and bled easily, and was covered with a yellowish flaky material which came off readily. Histological examination of the skin affected by this dermatitis revealed the following:

1. A marked thickening of the cornified layer to several-fold that of a normal skin.
2. A marked proliferation of the cells of the Malphigian layer to several-fold that of a normal skin.
3. Loosening of the underlying connective tissue, and its infiltration with blood elements proportionately higher in leucocytes. The connective tissue appeared also more highly vascularized.
4. Appearance of hemorrhages in the Malphigian layer.

It was also noted that in a normal chick the skin in the immediate neighborhood of the outlet of the uropygeal gland, as well as the lining of the ducts of the gland, showed thicker cornified and Malphigian layers when compared with the skin taken from other parts of the body.

Thus, an external application of fat to the skin of the chick caused a lethal dermatitis, whereas the incorporation of the same fat in the diet or its injection (subcutaneously) caused no apparent ill effects.

The histological studies were made under the guidance of Dr. Alexei Koneff of the Department of Anatomy, University of California.

11731

Effect of "Prospermin" on Immature and Mature Hypophysectomized and Normal Male Rats.*

J. H. LEATHEM. (Introduced by P. E. Smith.)

From the Department of Anatomy, College of Physicians and Surgeons, Columbia University, New York City.

The induction or restoration of sperm formation in mature and immature hypophysectomized ground squirrels by Prospermin† injections has been reported.[1] However, this material is stated to have only a slight effect on the normal rat testes.[2, 3] The present investigation was undertaken to determine the effect of Prospermin on sperm formation and on the interstitial tissue of hypophysectomized mature and immature male rats.

The majority of the immature male rats were operated at 24 to 28 days of age, a few being 30 to 37 days of age. A postoperative interval of 5 to 10 days was allowed to elapse after hypophysectomy before beginning treatment. The mature rats were 78 to 120 days old at the time of operation; the injections being begun after a postoperative interval of 0 to 60 days. All pituitary capsules were serially sectioned, stained with Masson trichrome and examined microscopically. Data from only completely hypophysectomized animals are presented.

The Prospermin was injected, subcutaneously, in daily doses of 1 and 2 mg. Higher dosages were not given as this extract was poorly

---

* Aided by a grant, administered by Dr. P. E. Smith, from the Rockefeller Foundation, New York.

† Prospermin is stated by the manufacturers, E. R. Squibb & Sons, to be prepared from normal male urine. This material was generously supplied by Dr. J. A. Morrell.

[1] Wells, L. J., and Overholser, M. D., *Anat. Rec.*, 1938, **72**, 231.

[2] Moore, C. R., *Am. J. Anat.*, 1936, **59**, 63.

[3] Morrell, J. A., *Anat. Rec.* (*Suppl.*), 1933, **55**, 29.

tolerated, even the 1 and 2 mg doses frequently necessitating the sacrifice of some of the animals before the planned period of treatment had expired.

Hypophysectomized Immature Males. Injection of 1 mg of Prospermin daily for 30 days induced sperm formation in the testes of 4 of 6 animals. However, the sperm were not as numerous as observed in the unoperated control animal and there was a marked increase of the interstitial tissue above that of normal males of the same age. Hypophysectomized control animals' testes were devoid of sperm. With the exception of one rat treated for 20 days, all of the 26 rats receiving 1 mg daily for 5 to 23 days failed to exhibit sperm in the testes but invariably had some interstitial tissue increase. Two mg of Prospermin were injected for 18 to 30 days in 7 rats. All had sperm in the testes. Very few sperm were observed after only 18 days of treatment, but they were numerous after treatment for 30 days. None of the 6 animals injected for less than 18 days had sperm although there was a stimulation of the interstitial tissue.

In all cases the testis and seminal vesicle weights were increased. This increase was greater in the longer than in the shorter treatments. After 30 days of treatment, the weights of the testes were approximately 10 times and those of the seminal vesicles 125 times those of hypophysectomized control rats. In no case were sperm present in the epididymis.

Hypophysectomized Mature Males. After a 10-day postoperative interval, either 1 or 2 mg daily doses were injected for 30-33 days in 7 hypophysectomized mature rats. Sperm were observed in the testes of all of the rats. However, the testis weights were but half that of the unoperated controls but the seminal vesicle weights were equal to the normal controls. After a 60-day postoperative interval, sperm were present in the testes of one of the 2 rats injected with 1 mg daily for 40 days.

In 9 mature males 0.5 and 1 mg daily was injected for 15 days, the injections being begun on the day of hypophysectomy. Testis weights and spermatogenesis were equal to those of unoperated littermate controls. The seminal vesicles were abnormally large.

Normal Immature Males. Normal male rats, 25 days of age, were injected for 15- and for 30-day periods with 1 mg of extract daily. Testis weights were not increased over those of littermate controls, a result which is in accord with that of Moore.[2] However, seminal vesicle weights were increased tenfold in both periods of treatment.

Summary. Hypophysectomized immature male rats responded to treatment with Prospermin by exhibiting testis enlargement and sperm formation. This extract did not increase testis weights in the unoperated immature animal. Restoration and maintenance of spermatogenesis was successfully accomplished in the hypophysectomized mature rat. A marked seminal vesicle weight increase was observed in nearly all of the treated animals.

11732

Immunological Distinction Between St. Louis and Japanese B Encephalitic Viruses.

Leslie T. Webster.

From the Laboratories of the Rockefeller Institute for Medical Research, New York City.

Experiments by Perdrau published posthumously indicated that St. Louis encephalitic virus, Strain No. 3, and Japanese B encephalitic virus, Strain No. 2, furnished by us, were immunologically identical.[1] These results are contrary to our previous findings[2] and hence have been checked in this laboratory.

Perdrau's technic was followed in preparing immune sera. The standard technic for setting up neutralization-tests used in both sets of experiments was continued. Serum-virus mixtures were tested after 4 hours according to our method, and again after 20 hours according to Perdrau's method.

We found at once that immune sera prepared as above[1] were equal or superior to ours in neutralizing capacity and that 20 hours' standing reduced the activity of the serum-virus mixtures one hundred- to one thousandfold, yet brought out any neutralizing effect more dramatically. Most important, however, was the ready confirmation of Perdrau's results with St. Louis No. 3 and Japanese No. 2 strains (Table I). Further tests in animals showed that the St. Louis strain had retained its distinguishing characteristics, whereas the Japanese virus behaved in every way like the St. Louis virus. Questions arose, therefore, as to whether (a) our previous findings[2] were erroneous, (b) the Japanese virus had become altered through repeated passage, or (c) the Japanese virus had at some time been erroneously labeled.

That an error had occurred in labeling was indicated by the following tests. Seven strains of virus were secured from outside

---

[1] Perdrau, J. R., *J. Path. and Bact.*, 1940, **50**, 545.

[2] Webster, Leslie T., *J. Exp. Med.*, 1938, **67**, 609.

TABLE I.

Complete Cross-Neutralization of St. Louis No. 3 and Japanese No. 2 Viruses.

Virus	Serum	Incubation hr	Fate of mice injected intracerebrally with serum-virus mixtures in dilutions					Neutralization in MLD
			10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	
St. Louis No. 3	Normal[*]	4	4/4[†]	4/4	4/4	4/4	*4/4*[§]	
	Equine encephalomyelitis		4/4	4/4	4/4	4/4	*4/4*	0
	Rabies		4/4	4/4	4/4	3/3	*4/4*	0
	St. Louis No. 3		4/4	4/4	*4/4*	1/4	0/4	10-100[‡]
	Japanese No. 2		4/4	4/4	*3/4*	1/4	0/4	10-100
	Normal	20	4/4	4/4	*4/4*	1/4	0/4	
	” brain		4/4	4/4	*4/4*	1/4	0/4	0
	Equine encephalomyelitis		4/4	4/4	4/4	*2/4*	0/4	0
	Rabies		4/4	4/4	4/4	*3/4*	0/4	0
	St. Louis No. 3		1/4	0/4	0/4	0/4	0/4	100+
	Japanese No. 2		1/4	0/4	0/4	0/4	0/4	100+
Japanese No. 2	Normal	20	4/4	4/4	*3/4*	0/4	1/4	
	” brain		4/4	4/4	*2/4*	1/4	0/4	0
	Japanese No. 2		0/4	0/4	0/4	0/4	0/4	100+
	St. Louis No. 3		0/4	0/4	0/4	0/4	0/4	100+
	” ” Broun ’33		0/4	0/4	0/4	0/4	0/4	100+
	” ” convalescent 434		*4/4*	1/4	0/4	0/4	0/4	10-100
	St. Louis convalescent 435		*4/4*	1/4	0/4	0/4	0/4	10-100
	St. Louis convalescent 437		4/4	*4/4*	0/4	0/4	0/4	1-10

[*] Normal = Normal rabbit serum.

Equine encephalomyelitis = Rabbit immunized with mouse brain equine encephalomyelitic virus.

Rabies = Rabbit immunized with mouse brain rabies virus.

St. Louis No. 3 = Rabbit immunized with mouse brain St. Louis No. 3 virus.

Japanese No. 2 = Rabbit immunized with mouse brain Japanese No. 2 virus.

Normal brain = Rabbit immunized with normal mouse brain.

[†] 4/4 = Four of 4 tested mice died from the experimental infection.

[‡] 1 MLD = Highest dilution fatal to 50% or more of controls.

[§] The dilution in italics signifies the titration-end point, 1 MLD.

TABLE II.

Partial Cross-Neutralization of St. Louis No. 3 and Broun 1933 Viruses.

Virus	Serum	Incubation hr	Fate of mice injected intracerebrally with serum-virus mixtures in dilutions					Neutralization in MLD
			10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	
St. Louis No. 3	Normal	20	4/4	4/4	*4/4*	1/4	0/4	
	” brain		4/4	4/4	*4/4*	1/4	0/4	0
	St. Louis No. 3		*4/4*	1/4	0/4	0/4	0/4	10-100
	” ” Broun ’33		*3/4*	0/4	0/4	0/4	0/4	10-100
St. Louis Broun ’33	Normal		4/4	4/4	*4/4*	0/4	0/4	
	” brain		4/4	4/4	*4/4*	0/4	0/4	0
	St. Louis Broun ’33		1/4	0/4	0/4	0/4	0/4	100+
	” ” No. 3		4/4	*4/4*	0/4	0/4	0/4	1-10

See Table I for explanation of footnotes.

TABLE III.
Lack of Cross-Neutralization Between St. Louis and Japanese B Encephalitic Strains.

Virus	Serum	Incubation hr	Fate of mice injected intracerebrally with serum-virus mixtures in dilutions 10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	Neutralization in MLD
St. Louis No. 3	Normal	20	4/4	*4/4*	0/4	0/4	0/4	—	
	St. Louis No. 3		0/4	0/4	0/4	0/4	0/4	—	10+
	Japanese Nakayama		4/4	*3/4*	1/4	0/4	0/4	—	0
Japanese Nakayama	Normal	4	4/4	4/4	4/4	4/4	*4/4*	1/4	
	St. Louis No. 3		4/4	4/4	4/4	4/4	*4/4*	1/3	0
	St. Louis convalescent		4/4	4/4	4/4	4/4	*3/4*	0/3	0
	Normal	20	4/4	4/4	3/4	*2/4*	1/4	0/4	
	St. Louis No. 3		4/4	4/4	4/4	*2/4*	0/4	0/4	0
	St. Louis convalescent		4/4	4/4	*3/4*	0/4	0/4	0/4	1-10
Japanese Nakayama	Normal	20	4/4	*4/4*	0/4	0/4	0/4	0/4	
	Nakayama		0/4	0/4	0/4	0/4	0/4	0/4	10+
	St. Louis No. 3		*2/4*	0/4	0/4	0/4	0/4	0/4	1-10
Jap. Sasaki	Normal	4	4/4	4/4	4/4	*3/4*	0/4	0/4	
	St. Louis No. 3		4/4	4/4	*4/4*	1/4	0/4	0/4	1-10
	Normal	20	4/4	4/4	*2/4*	1/4	0/4	0/4	
	St. Louis No. 3		4/4	*2/4*	0/4	1/4	0/4	0/4	1-10
Jap. Sakurai	Normal	20	4/4	*4/4*	0/4	0/4	0/4	0/4	
	Japanese Nakayama		0/4	0/4	0/4	0/4	0/4	0/4	10+
	St. Louis No. 3		4/4	*4/4*	0/4	0/4	0/4	0/4	0
Jap. Sawatani	Normal	20	4/4	4/4	*2/4*	0/4	—	—	
	Japanese Nakayama		0/4	0/4	0/4	0/4	—	—	100+
	St. Louis No. 3		4/4	*3/4*	0/4	0/4	—	—	1-10

			10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	
Jap. Calinina	Normal	20	—	4/4	*4/4*	1/4	0/4	—	—	
	Japanese Nakayama		0/4	0/4	0/4	0/4	—	—	—	100+
	St. Louis No. 3		4/4	*3/4*	1/4	0/4	—	—	—	1-10

See Table I for explanation of footnotes.
— = Dilution not tested.

sources. Dr. G. O. Broun kindly supplied St. Louis strains from the 1933 and 1937 epidemics, Dr. E. H. Lennette, the Japanese strains Nakayama and Sasaki, Dr. R. Kobayashi, the Japanese strains Sakurai (1939) and Sawatani (1939), and Dr. T. Mitamura the Japanese strain Calinina (1935). These viruses proved free of lymphocytic choriomeningitic virus.

In animals the strains behaved according to previous descriptions[2] except that the response of *Macacus rhesus* monkeys to the Japanese strains was somewhat less explosive.

Immune rabbit sera were prepared with the St. Louis Broun 1933 strain and the Japanese Nakayama strain.

Neutralization tests shown in Table II indicate that St. Louis No. 3 virus was neutralized equally well by homologous and St. Louis Broun 1933 immune sera (10 to 100 M.L.D.), but that the Broun strain was less effectively neutralized in the St. Louis No. 3 (1 to 10 M.L.D.) than in the homologous immune sera (10 to 100 M.L.D.). Thus there appeared slight immunological differences between the 2 St. Louis strains.

Table III shows that (1) St. Louis No. 3 virus was not neutralized by the Japanese Nakayama immune serum and (2) the 5 Japanese strains were neutralized either not at all or only in the M.L.D. dilution by St. Louis No. 3 immune serum and human St. Louis convalescent sera.

Mice immunized with St. Louis No. 3 virus were not immune to as little as 1 M.L.D. of the Nakayama virus.

Thus, on present evidence, we regard the relationship between St. Louis and Japanese B virus to be as stated in 1938.[2] They are closely related with respect to the epidemiology of the diseases which they produce. Together with louping-ill and equine encephalomyelitic viruses, they possess similar filterability-characteristics with Elford membranes. In animal species, St. Louis, Japanese, and louping-ill viruses show general similarity and Japanese B and louping-ill viruses appear identical. And finally, St. Louis, Japanese, and louping-ill viruses, in spite of an occasional cross-neutralization not exceeding 1 to 10 M.L.D., remain immunologically readily distinguishable.

11733

Androgenic Assay of the Human Fetal Adrenal.*

WILLIAM H. CARNES. (Introduced by J. W. Jobling.)

From the Department of Pathology, College of Physicians and Surgeons, Columbia University, New York City.

It is indicated from the study of castrate men and women that there is an extragonadal source of sex hormones of some abundance. This was suspected to be the adrenal long before the actual demonstration by Reichstein[1] that small amounts of androgenic material could be chemically isolated from beef adrenals. The strongest reason for suspecting the adrenal as a source of these hormones has been the occasional occurrence of sexual changes (the "adrenogenital" syndrome)[2] and the urinary excretion of large excesses of some of these hormones[3, 4, 5] in individuals harboring a neoplasm of the adrenal gland (and the regression of the sexual changes and diminution of the urinary hormone excretion following surgical removal of the tumor in some of these cases). Marked bilateral hyperplasia of the adrenal cortex is associated with other cases of virilism in women and is the almost invariable accompaniment of congenital female pseudohermaphroditism.[6] Furthermore, androgenic hormone has been found in the adrenal tissue itself in a case of macrogenitosomia praecox[7] with excessive hyperplasia of the adrenals. The majority of these observations, therefore, suggest androgenic more than estrogenic secretion by the adrenal. Nevertheless, adrenal cortical tumors and some degree of cortical hyperplasia are common and only a very few of these cases have associated sexual changes. Grollman[2] has made an attractive suggestion that this is due to the existence of 2 distinct functional types of cell in the human adrenal cortex, the one concerned with producing a vital hormone and the other with producing an androgenic hormone. The latter function he believed to reside in the great bulk of cortical tissue composing most of the gland in fetal life which promptly and regularly involutes after birth.[8] The persistence of a few cells of this fetal tissue has been postulated in the juxta-medullary zone of the gland even into adult life[9] and it is from these cells that Grollman has suggested that those tumors and hyperplasias influencing the sexual characters arise. There is some support for such a hypothesis on morphological grounds[9, 10] although it is admitted that no rigid histological criteria are available for establishing it.

---

* The autopsy material for this study was collected at the Baltimore City Hospitals and the Johns Hopkins Hospital, Baltimore.

[1] Reichstein, T., *Helv. Chim. Acta*, 1936, **19**, 223.

It was with the hope of adding some evidence on this hypothesis that adrenal tissue was collected from human fetuses for biological assay. The material was obtained from 20 stillborn and newborn† fetuses autopsied at from 4 to 44 hours postmortem. The fetuses varied from 18 to 40 weeks in period of gestation. Approximately 100 g of wet adrenal tissue was obtained. The glands were chopped up and preserved in neutral acetone in tightly covered bottles in the cold. The preliminary extraction was accomplished by 2 changes of this acetone, 10 and 5 fold excesses respectively, and 2 additional excess quantities of ethyl ether for 24 and 48 hours respectively. The acetone fractions were combined and the acetone removed at less than 40°C under reduced pressure. The residue from this was taken up in ether and all the ether fractions combined and dried with solid anhydrous sodium sulphate. About 40% of the ether extract was condensed into a small convenient volume of olive oil by evaporation on the steam bath-(A). This method is the same as that employed by Parker and Tenney[11] in their study of the estrogenic content of the tissues in pregnancy except that the additional precaution of vacuum distillation was used in removing the acetone in order to keep the temperature under 40°C.

---

[2] Grollman, A., *The Adrenals,* Williams and Wilkins Co., Baltimore, 1936.

[3] Frank, R. T., *J. A. M. A.,* 1937, **109**, 1121.

[4] Koch, F. C., *Ann. Int. Med.,* 1937, **11**, 297.

[5] Friedgood, H. B., and Whidden, H. L., *New England J. Med.,* 1939, **220**, 736.

[6] Young, H. H., *Genital Abnormalities, Hermaphroditism, and Related Adrenal Diseases,* Williams and Wilkins Co., Baltimore, 1937.

[7] Wilkins, L., Fleischmann, W., and Howard, J. E., *Endocrinology,* 1940, **26**, 385.

[8] Elliott, T. R., and Armour, G., *J. Path. and Bact.,* 1911, **15**, 481.

[9] Goormatigh, N., *Le cortex surrénal humain dans les plaies de l'abdomen et aux périodes intéressantes de la vie sexuelle,* Liege, 1922.

[10] Broster, L. R., and Vines, H. W. C., *The Adrenal Cortex,* London, 1933.

The remaining 60% of the ethereal solution was extracted with 5 small portions of 10% aqueous NaOH to remove the estrogenic substances, then washed with several changes of distilled water, dried again with anhydrous sodium sulphate and condensed into a convenient volume of olive oil on the steam bath-(B).

The method of assay for androgenic activity was a slight modification of the chick comb test[12] recommended to me by Dr. Ralph B. Oesting. The oily solution was directly applied to the comb with a pipette. Day-old chicks were used. A solution of testosterone propionate in oil was the standard of comparison. Estrogenic assays were done by the immature rat uterus method.[13] Crystalline estrone in oil was the standard of comparison.

Fraction A was first assayed for both androgen and estrogen. No androgen was detected but the estrogen content was estimated as equivalent to 272 γ of estrone per kilogram of fresh tissue (2720 I.U. estrogen per kg). Fraction B was therefore prepared in order to have an androgen fraction free of estrogen since it is known that estrogen may inhibit the comb response in some degree. The androgenic assay on Fraction B was likewise negative in such quantity as to indicate a concentration less than equivalent to 1 mg testosterone propionate per kilo of fresh tissue. This indicates an androgenic content (if any) of less than 10 I.U. of activity per kilo of fresh adrenal.‡

---

† To avoid including involuted fetal cortex, no glands were used from babies living more than 48 hours after delivery. Obviously autolyzed specimens were not included.

[11] Parker, F., Jr., and Tenney, B., Jr., *Endocrinology*, 1938, **23**, 492.

[12] *a*. Dorfman, R. I., and Greulich, W. W., *Yale J. Biol. and Med.*, 1937, **10**, 79; *b*. Frank, R. T., Klempner, E., and Hollander, F., PROC. SOC. EXP. BIOL. AND MED., 1938, **38**, 853.

[13] Lauson, H. D., Heller, C. G., Golden, J. B., and Sevringhaus, E. L., *Endocrinology*, 1939, **24**, 35.

The significance of these observations is admittedly limited. A positive result would have permitted more positive conclusions. All that can be said is that no support has been found for the hypothesis that the human fetal adrenal produces androgenic hormone. The technic of extraction and assay is well standardized and controlled. Some degree of autolysis of the tissue before extraction was inevitable but previous experience indicates that this would be no detriment.[11] It would be vain to conjecture what concentration of hormone should be expected in a tissue functionally androgenic. The hormone content of the endocrine glands is known to vary widely under different physiological circumstances. The hyperplastic adrenal in the case of macrogenitosomia praecox mentioned above[7] contained an estimated 715 I.U. of androgen per kilo of fresh tissue.

The estrogen concentration found was similar to that found in the fetal adrenal by Parker and Tenney[11] in their study of the estrogen content of several maternal and fetal tissues in pregnancy. As these authors have demonstrated, this cannot be taken as evidence of the site of formation of estrogenic hormone in this instance, since in pregnancy all the tissues of both mother and fetus contain large amounts of the hormone. The demonstration of estrogen in this tissue, however, does afford some control of the efficacy of the extraction.

Summary. In spite of some evidence suggesting an androgenic function of the human fetal adrenal cortex, it has not been possible to demonstrate any androgenic activity by biological assay of the tissue by a method adequate to detect as little as 10 I.U. per kilo of fresh tissue.

---

‡ Repeated comparisons have shown that by this method of assay testosterone propionate has an approximately equal activity to the same weight of cis-androsterone, the international standard.

11734

Anti-Gonadotropic Substances in Man Following Treatment with Pregnant Mare Serum.*

JOSEPH W. JAILER AND JAMES H. LEATHEM. (Introduced by Earl T. Engle.)

From the Department of Anatomy, College of Physicians and Surgeons, Columbia University, New York City.

The appearance of hormone antagonists in the blood serum as a result of treatment with hormone preparations was discovered by Collip and Anderson.[1] Although preparations of gonadotropic substances from the anterior pituitary gland, pregnant mare serum and human pregnancy urine have been used extensively in the clinics, little consideration has been given to the demonstration of inhibitory substances appearing in the human. The presence of inhibitory substances in the serum following injections of thyrotropic preparations has been reported[2] and similar findings were obtained in 9 patients, with undescended testes, following treatment with massive doses of pregnant mare serum.[3]

The purpose of this study was to determine whether anti-gonadotropic substances appeared in the blood of female patients undergoing treatment with pregnant mare serum gonadotropin (PMS). We wish to express our appreciation to Dr. C. L. Buxton for making this work possible by placing patients from the clinic of the Sloane Hospital for Women at our disposal.

Three women, age 22 to 34, suffering from sterility which was accompanied by dysmenorrhea in 1 case, were treated with a total of 4,800 to 8,900 r.u. (Cole-Saunders) of PMS (Gonadin†) during a period varying from 4 months to over a year. In this group the injections were given only during the middle of the menstrual cycle in an attempt to induce ovulation. One patient with amenorrhea of 10 years' duration served as an uninjected control.

---

* Aided bv a grant, administered by Dr. P. E. Smith, from the Rockefeller Foundation, New York.

[1] Collip, J. B., and Anderson, E., *Lancet*, 1934, **1**, 76.

[2] Collip, J. B., and Anderson, E., *J. Am. Med. Assn.*, 1935, **104**, 965.

[3] Rowlands, I. W., and Spence, A. W., *Brit. Med. J.*, 1939, **2**, 947.

† Mare serum hormone "Gonadin" was generously supplied by Dr. Donald Wonder, Cutter Laboratories, Berkeley, California. An international unit of mare's serum gonadotropin is the equivalent of 0.25 mg of the international standard. The Cole-Saunders rat unit is said to be the equivalent of 0.5 mg or twice that of the international standard.

Two male patients who were treated for hypogonadism received a single injection of 200 r.u. of PMS each week for 7 and 9 weeks.‡

As test animals, 21-22-day-old female mice were used. All test mice received a total of 5 r.u. of PMS (Gonadin) and, at a different site, 0.9 or 1.0 cc of the serum to be tested. Injections were made subcutaneously once daily for 3 days and the animals sacrificed 72 hours after the first injection. The weights of the ovaries and uteri were obtained, the latter after removal of intra-uterine fluid. In all, the blood from 7 patients was analyzed for anti-gonadotropic activity. The blood was kept in the refrigerator and the serum test started 24-48 hours after the blood sample was taken.

The results are summarized in Table I, where it is shown that 0.9 or 1.0 cc of serum from patients Nos. 1, 2 and 3 completely inhibited the gonadotropic action of 5 r.u. of PMS. These patients had received a total of 4,800 to 8,900 r.u. during a period of as

TABLE I.
Anti-gonadotropic Activity of Serum from Individuals Following Mare Serum Hormone.

Patient (age)	Condition	Treatment	No. mice	Injections PMS	Injections Serum	Avg ovar. wt mg	Avg uter. wt mg	Neutralization
1 ♀ (25)	Steril.	2/10/39-4/5/40 8,900 r.u.	4	5	1.0	3.2	8.6	Complete
			3	5	0.9	2.8	18.9	”
2 ♀ (22)	Steril. dysmenorrhea	6/21/39-3/1/40 7,200 r.u.	7	5	0.9	2.4	6.3	”
3 ♀ (34)	Steril.	Oct.-Dec. 2,400 r.u. Jan.-Feb. 2,400 r.u.	5	5	0.9	2.6	7.5	”
4 ♀ (21)	Steril. dysmenorrhea	4/17-5/5 1,000 r.u.	2	5	0.9	5.4	35.0	Partial
7 ♀ (26)	Amenorrhea	None	4	5	0.9	9.0	32.1	None
5 ♂ (40)	Hypogonadism	7 wk injections 200 r.u. each	6	5	0.9	11.5	33.2	”
6 ♂ (31)	Hypogonadism	9 wk injections 200 r.u. each	6	5	0.9	6.5	49.6	Partial
		Control Experiments.						
			26	5	None	9.2	34.1	
			10	None	”	3.1	7.9	

---

‡ We wish to thank Dr. G. W. Fish for placing the male patients at our disposal.

long as a year. These inhibitory substances remain in the blood long after cessation of treatment; blood was collected 4 months after the last injection (Patient No. 3), and yet 0.9 cc of serum completely inhibited 5 r.u. of PMS.

Patient No. 4 (sterility accompanied by dysmenorrhea) received a total of only 5 injections (1,000 r.u.) of PMS, given over a 3-week period. Inhibitory substances were definitely present in the blood 10 weeks after the first treatment.

Patient No. 7 received no treatment and antigonadotropic substances were not detected.

Treatment with Synapoidin,§ following PMS administration, did not interfere with the production of anti-serum to PMS in the one case (No. 1) tested. Furthermore, anti-gonadotropic activity to the Synapoidin was not evident 6 weeks after the first of 14 daily injections.

Summary and Conclusions. The injection of gonadotropic substances of equine origin elicits the production of anti-gonadotropic activity in man. It has been reported previously[3] that daily or biweekly administration of PMS caused anti-gonadotropic serum formation. The data reported here demonstrates that PMS treatment extending over a period of months will build up anti-gonadotropic activity despite the fact that hormone therapy involved only a few injections (4-5) at each mid-period of the menstrual cycle. Furthermore, this activity persists for as long as 4 months after the cessation of treatment.

Definite anti-gonadotropic activity was evident in the serum of patients receiving shorter treatment. The time factor is of importance since anti-serum of the same potency resulted after 9 injections (200 r.u. each) given once a week and after 5 injections (200 r.u. each) administered in 3 weeks. The serum was tested 10 weeks after the first injection in each case.

It is interesting to note that inhibitory substances to human pregnancy urine extracts have never been detected in man,[4, 5, 6] whereas they have been shown to occur after treatment with heterologous extracts. This fact supports the contention that the inhibitory substances are antibody in nature, elicited by an extract of a different species, rather than anti-hormone to the injected active material.

---

§ Synapoidin consists of pregnancy urine plus Evans' pituitary synergist and was kindly supplied by Parke, Davis & Co.

[4] Sulman, F., *J. Exp. Med.*, 1937, **65**, 1.

[5] Spence, A. W., Scowen, E. F., and Rowlands, I. W., *Brit. Med. J.*, 1938, **1**, 66.

[6] Dorff, G. B., *Endocrinology*, 1938, **22**, 669.

11735

Hemolytic Streptococci from the Throat of Normal Monkeys.

A. Pomales-Lebrón and P. Morales-Otero.

From the Department of Bacteriology of the School of Tropical Medicine, University of Puerto Rico, San Juan, P. R.

In November 14, 1938, a shipment of 500 Rhesus monkeys arrived in San Juan from India. These animals came, on the average, from 300 miles inland from Calcutta. The period of time they remained in this city awaiting shipment varied with different animals from a few days to 3 weeks. The shipment left Calcutta, September 30, 1938. The ports touched enroute were: Colombo, Ceylon, one day; Boston Mass., 2 days; and New York City, 2 days.

We studied the throat flora of 172 of these monkeys soon after arrival, over a period from November 20 to December 12, 1938, and the beta-hemolytic streptococci recovered were studied in considerable detail. The materials and methods used were the same as those employed in a previous investigation.[1]

During the second week of December, 1938, the monkeys were taken to Santiago* Primate Colony, where they were set free. Throat cultures from 309 monkeys which were in apparent good health were taken again over a period from January 16 to February 22, 1940, and the hemolytic streptococci recovered were studied, utilizing media and methods similar to those employed before.

Seegal, Heller and Joblanowitz[2] studied the normal bacterial flora of 48 monkeys in New York City and recovered beta-hemolytic streptococci in 28 instances. Of these, 19 were Group A, 4 Group C, 5 Group G, and one, a minute streptococcus belonging to Group F.

Of the 172 monkeys examined in 1938, 22 (12.7%) harbored hemolytic streptococci in their throat, of which 10 (45.6%) were Group A, 8 (35.9%) were Group C, and 4 (18.5%) were Group G.

Of the 309 monkeys examined in 1940, 39 (12.5%) harbored hemolytic streptococci in their throat. None of them were Group A. Twenty-six (66.6%) were Group C, and 13 (33.3%) were Group G. It is interesting to note that, though the incidence of beta-hemolytic streptococci is the same in both cases, about one-half of the streptococci recovered from the throats of the monkeys soon after arrival, belonged to Group A, while no streptococci belonging to this serological group were encountered among the strains obtained after the monkeys had been in Santiago Island for about 14 months.

---

[1] Pomales-Lebrón, A., *P. R. J. Pub. Health and Trop. Med.*, 1940, **16**, in press.

* This is a small island (about 35 acres) approximately one mile off the eastern coast of Puerto Rico.

[2] Seegal, B. C., Heller, G., and Joblanowitz, J., Proc. Soc. Exp. Biol. and Med., 1936, **34**, 812.

The average final pH in glucose broth in both series varied from 5.1 to 5.3. None of the Group A strains gave reduction of methylene blue in milk. Of 8 type C strains isolated in 1938, 7 (87%) reduced methylene blue. Of 26 type C strains isolated in 1940, 23 (88%) reduced methylene blue. Of 4 type G strains of the first series, 3 (75%) reduced methylene blue and out of 13 strains of the second series only 3 (23%) reduced the dye.

According to Hollman's classification, 17 strains of those isolated in 1938 were *pyogenes,* 10 of these belonging to Group A and 7 to Group C. Five were *equi;* of these, one belonged to Group C and 4 to Group G. None fell in the *subacidus* group.

In the 1940 series, 4 strains fell in the *pyogenes* group, 2 belonging to Group C and 2 to Group G. Twenty-seven fell in the *equi* group; 17 of these were Group C and 10 Group G. Eight belonged to the *subacidus* group; 7 of these were Group C and one was Group G.

Out of the 22 strains isolated in 1938, 21 were fibrinolytic against human plasma clot, the average dissolution time being 1.9 hours. All these strains were only very slightly fibrinolytic for Rhesus monkey plasma in 24 hours. Out of 39 strains isolated in 1940 31 were fibrinolytic to human plasma clot. Eight strains, belonging to Group C, were not fibrinolytic. All strains were very slightly fibrinolytic for Rhesus monkey plasma.

Seegal, *et al.,*[2] also noted the relative insusceptibility of monkey plasma to the lytic action of their strains, which, however, were active against human plasma clot.

The virulence for mice was tested by injecting 0.5 cc of a 20-hour glucose (.02%) neopeptone broth culture intraperitoneally. Of the 22 strains isolated in the first series, 11 (50%) killed mice in less than 4 days, and of the 39 strains isolated in the second series, 36 strains (92%) killed mice in less than 4 days.

Hemolysis was tested with the whole culture and with the supernatant after centrifugation and reduction with sodium hydrosulphite. Streptolysin broth[3] was used in these experiments. Using the whole culture, all strains of both series showed positive hemolysis. Using the supernatant after centrifugation and reduction, in the first series, out of 10 Group A strains, 4 showed a reducible hemolysin and all the 12 strains belonging to Groups C and G showed hemolysis. Out of 39 strains of the second series belonging to Groups C and G, 32 showed a reducible hemolysin. All the Group C and G strains failed to produce an erythrogenic toxin reactive on the skin of a susceptible white goat, and of the 10 Group A strains, 4 produced an erythrogenic skin toxin, 3 gave a doubtful reaction and 3 were negative. None of the strains studied hydrolyzed sodium hippurate. All except one fermented trehalose and did not ferment sorbitol. The exception was a Group C strain (sorbitol positive; trehalose negative), which produced a good beta-hemolysis in blood agar but gave a negative tube hemolysis both in the whole culture and with the supernatant after reduction with sodium hydrosulphide. It was non-fibrinolytic when tested against human or monkey plasma, and was classified as *Streptococcus pyogenes* (Hollman).

---

[3] Swift, H. F., and Hodge, B. E., PROC. SOC. EXP. BIOL. AND MED., 1933, **30**, 1022.

Discussion. The biological characteristics of the organisms studied seem to prove that the hemolytic streptococci found in the throats of normal monkeys are of human origin.

The incidence of hemolytic streptococci in the normal throats of these monkeys is higher than that in the throats of normal humans in this island.[3]

It is of interest to note that Group A hemolytic streptococci were not found in the throat of monkeys after these had been living in the island for more than a year. This is in accord with previous findings in normal human beings living in the island, which show that the incidence of Group A strains among the beta-hemolytic streptococci isolated from normal throats and from throats in the general population of the island of Puerto Rico is lower than the incidence among strains cultured from similar sources in temperate climates.[1] These findings may help to explain the low incidence of certain streptococcal conditions in Puerto Rico.

inhibition of the proliferation of the organisms in the tissue.

In order to corroborate these results, a second experiment was made.

Experiment II. A stock culture of the same strain employed in Experiment I was inoculated into a mouse and the organisms recovered from the heart's blood were used in the preparation of a saline suspension which was adjusted to the same turbidity as before.

Sixty mice were utilized, divided into 3 groups as follows:

Group A: Sulfanilamide plus organisms.
Group B: Sulfamethylthiazol plus organisms.
Group C: Organisms alone.

The mice were given a single dose (0.5 cc) of the drug by mouth and immediately inoculated intraperitoneally with 0.1 cc of the *Brucella melitensis* suspension. Groups A and B were kept thereafter on a diet containing one percent of the drug.‡ Group C was kept on a diet of Purina dog chow and water.

Two weeks after initiating the experiment, 9 animals in Group A, 7 in Group B, and 18 in Group C had died. The great majority of animals could not be autopsied due to advanced decomposition. Three animals in Group B (2, 4 days, and one, 7days after inoculation) were the only cases autopsied, and in all 3 the specific organism was recovered from the blood. Thirty-six days after inoculation, the animals still alive (11 in Group A, 13 in Group B, and 2 in Group C) were killed and autopsied and cultures made from the heart's blood and splenic tissue in every instance. The amount of splenic pulp used for culture was approximately the same in all cases. Material was smeared on the surface of freshly prepared tryptose

TABLE I.
Results of Cultures of Blood and Splenic Tissue of the Mice Which Survived the Experimental Period of 36 Days.

Treatment	No. of animals	Spleen		Blood	
		Positive	Negative	Positive	Negative
Sulfanilamide	11	10*	1	0	11
Sulfamethylthiazol	13	8†	5	0	13
None	2	2‡	0	0	2

*In one case growth was abundant. In the rest, only few colonies on slant.
†Few colonies in all.
‡Abundant growth in both instances contrasting markedly with the comparatively poor growth obtaining from the treated animals.

---

‡ Purina dog chow was ground to a fine powder. The sulfanilamide was also ground to a fine powder and intimately mixed with the food. The same was done with the sulfamethylthiazol which was supplied to us in powder form.

agar slants and incubated aerobically during 4 days at 37°C. The results are summarized in Table I.

Comments. Sulfamethylthiazol is more effective than sulfanilamide in the treatment of experimental Brucella infection in mice. The results suggest that the proliferation of the organisms is only partially inhibited by treatment with a tendency towards the establishment of a chronic infection. These drugs may be of use in the study of chronic infection in the experimental animal.